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Investigating Estrogenic Endocrine Disrupting Compounds and Their Disinfection Byproducts Within Drinking Water Treatment

Kirsten E. Studer

INVESTIGATING ESTROGENIC ENDOCRINE DISRUPTING COMPOUNDS AND THEIR DISINFECTION BYPRODUCTS WITHIN DRINKING WATER TREATMENT

A Project Presented

by

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DEDICATION

To Kenneth Studer, my father

To Kevin and Kathryn, my brother and sister

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ABSTRACT

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Emerging public health concerns relating to the epigenetic effects of EDCs, along with the reconceptualization of dose response curves, provides a compelling rationale for addressing estrogenically active contaminants in drinking water. These environmental health concerns are now known to have long lasting impacts, especially on fetal development. For this drinking water research, the estrogenic EDC byproducts were identified and the treatment processes were compared using the dose applied, the number of byproducts formed and the relative quantification of the treatment byproducts. The analytical optimized method presented and implemented in this research successfully determined the percent degradation of the parent compound for each disinfection treatment selected. From the resulting data, the chlorination of EE2 and DES produced the highest percent degradation of the parent compound, with the least number of byproducts. The optimized method decreased sample variability; showed a better fit to a linear calibration with both high and low concentrations of the parent compound; and lower MQLs and MDLs. Continuing research is needed to help in understanding the complete consequences of estrogenic endocrine disruptors in drinking water and the inevitable public health impact.

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CHAPTER 1 Introduction

1 Introduction

1.1 Overview of the Endocrine Disruptor Problem

One group of drinking water contaminants that has been of particular world-wide concern is endocrine disrupting chemicals or EDCs. The present research builds on and extends studies of EDCs that demonstrate the potential dangers of low level chemical interactions and by-product formation produced by traditional decontamination techniques (Huber *et al.*, 2004; Petrovic *et al.*, 2004; Richardson, 2002). A kinetic model for chemical decontamination processes is, therefore, required to determine the behavior and complexity of the chemical system. This includes not only direct chemical analysis but simulation of chemical processes that may have an adverse future impact on the water system. Such kinetic models will provide the foundation for rapid response required to rectify compromised water systems, whether this has been caused by increased use of biologically active compounds, natural disasters, or other situations.

EDCs are anthropogenic chemicals, which disrupt the endocrine system of animals and humans, even when the exposure dosage is low. The United States Environmental Protection Agency (USEPA) defines endocrine disrupting chemicals as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (EPA, 2010). The definition that the USEPA provides only allows external sources to be designated as EDCs; however, external factors are not the only factors in an organism's environment that can act as EDCs. In addition to the exogenous sources, the internal hormonal system can be altered in a way that can propagate endogenous effects from previous exogenous

exposures, with biological pathways being activated and inactivated at inappropriate times; therefore, disrupting the endocrine system (Birnbaum and Fenton, 2003). Such second-order effects can initiate long term systemic disruptions.

The endocrine system is critical because it works in parallel with the nervous system to direct growth and maturation (Thomas *et al.*, 1992). It is also important because it manages, regulates, and coordinates a constant internal environment, through homeostasis, by sending chemical messages to the appropriate receptor cells. Estrogens are one type of chemical messengers, or hormones that promote and maintain the development of the female secondary sex characteristics within the endocrine system (Farabee, 2001). Because the endocrine system is a delicate balance of complex chemical pathways, it is not always apparent from observing the outcomes that the overall effect is proportional to the level of disruption.

According to Gilbert and Epel, endocrine disruption is a functional change to tissue that appears superficially normal (2009). The pathology may be evident only in minute inspection and/or it may manifest solely as an alteration in gene expression. The sensitivity to endocrine disruption depends on the developmental stage, dose of the endocrine disruptor, and sex of the exposed entity. With the addition of a disruptor, there may be an additive or synergistic effect with nutritional and genetic background influences. The relative effect of endocrine disruption can span generations due to morphogenesis leading to dysfunctional physiology, which can be transmitted to the next generation (Gilbert and Epel, 2009; Norris and Carr, 2006).

1.2 Exposure to Endocrine Disruptors

Endocrine disruptors are introduced to the environment through many venues, as discussed in Section 1.6.1 *Source of EDCs in the Environment*. Once these contaminants are in the environment, they are difficult to detect and often impossible to avoid. Since endocrine disruptors are active at very low levels, the amounts found in the environment are often deemed harmless yet have potential implications to the functioning of the body's endocrine system.

The endocrine system is very complex and has many pathways that can be disrupted internally (endogenously) and externally (exogenously). Complex systems are characterized by such interactions that lead to the emergence of new relationships at different levels of chemical organization. The endocrine system, as a complex system, is composed of ductless glands that secret various hormones into the vascular system which are then transported to distant target tissues, where the hormones produce specific effects (Norris and Carr, 2006). Chemical regulators are also released into the vascular system by the nervous system (neurohormones) to regulate the release of hormones. This activity typically takes place within the liver, heart, kidneys and adipose tissue (Brown, 1994).

Endocrine disruptors can also interrupt other pathways in the body and are not limited to the endocrine system. The signals between cells, within cells, between organs and between organisms are all vulnerable to endogenous and exogenous disruption (Colburn, 1997).

Human exposure to these chemicals in food, water and the environment is a growing critical concern with unknown long-term and multi-generational impacts. Humans have evolved

chemical defenses that help protect against some natural and synthetic endocrine disruptors; however, natural compounds are more likely to be eliminated by the biological defense system (Colborn, 1997). Synthetic EDCs are more likely to be overlooked by the body's defense mechanisms because they mimic the natural compounds activity in the normal biological pathways.

Some EDCs have been linked to birth defects, infertility, immune system suppression, deformities to the reproductive organs, and various other health problems (McLachlan, 2001). Exposure to EDCs, such as estrogenic EDCs, has been implicated in the observed decrease in sperm counts in human males (McLachlan *et al.*, 1996; Miljoproekt, 1995). More significantly, the developing fetus is exceptionally sensitive to both the natural hormone signals used to guide its development, and the exogenous chemical signals that reach it from the environment. These natural signals and exogenous "morphogens" guide the fetus through its developmental path and help set the sensitivity to subsequent hormonal signals. This involvement of setting sensitivity can have life-long and multi-generational consequences.

Some of the synthetic chemical compounds are even more worrisome due to their accumulation in fatty tissues within the body (Langston, 2010). These EDCs can be passed on in the womb and through breast milk to infants at critical stages in their growth cycles. The chemical accumulation in the fat tissue and breast milk can reach very high concentrations over time, based on exposure type. In humans, the metabolic half-lives of estrogenic EDCs estradiol and ethinylestradiol are approximately 20 min and 17 h, respectively (Bolt *et al*., 1996). Even

though these half-lives are relatively short, there may be long term effects associated with such exposures.

Early developmental stages through puberty are especially vulnerable to chemical exposure due to the rapid changes that occur during this time, which are dramatically enhanced compared with that in an adult. A fetus changes quickly, with rapid cycles of cell division and growth, and massive changes in the patterns of gene activation over time. Exposure to EDCs can initiate a process of “flipping switches” on and off (DNA methylation) at inappropriate times during sexual development, which can lead to transgenerational epigenetic effects. With these numerous changes, there is ample opportunity for disruption leading to mistakes in developmental transcription. Comparable periods of cell division, differentiation and growth do not occur during adulthood; therefore, the opportunity for disruption of the pathways is substantially less compared to the prenatal through puberty period.

Another reason for the susceptibility toward disruption in fetal systems stems from the incomplete development of the blood-brain barrier when still in the womb (Gilbert and Epel, 2009). A second physiological barrier that is incomplete in fetuses is the enzymatic mechanisms that help to eliminate contaminants (Birnbaum et al., 2003). Therefore, fetal effects from maternal exposure at critical stages of development can have adverse effects on the fetus and subsequent generations but not have any effect on the mother. Figure 1-1 shows the specific sensitivities of an embryo/fetus to "morphogens," referred to as terotogens.

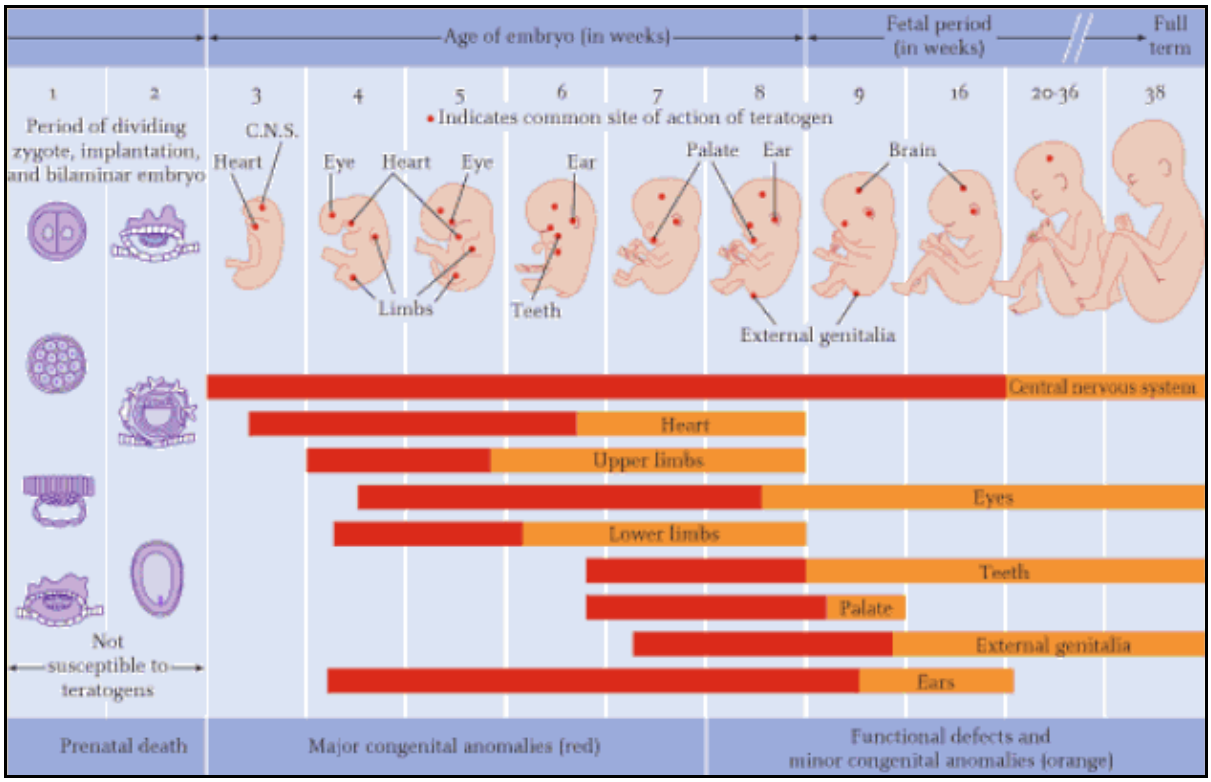


Figure 1-1: Developmental Sensitivity to Teratogens (Moore and Persaud, 1993)

Experimental evidence from animals shows that exposure to endocrine-disrupting compounds in early development can cause cancer and/or increase vulnerability to cancer-causing agents later in life (Birnbaum et al., 2003). Almost all human epidemiological research into cancer risk from contaminant exposures examines contaminant levels at the time of diagnosis or afterward; therefore, the assessment fails to include an entire period of developmental sensitivity to exposures that animal studies have identified. The critical exposure window may be much earlier than when the current dose response measurement is made if the early stages of life are critical to disease susceptibility.

Many of the exposure effects may not be recognizable or quantifiable until many generations later. Epigenetic impacts from exposure to EDCs are a very real consequence for their presence in the environment. Some responses already point to these contaminants as the reason for decreased sperm counts and increased cases of breast cancer (Melmed *et al.*, 2008; vom Saal *et al.*, 1995).

Another issue is the synergistic effects stemming from exposure to multiple contaminants. Research has shown that the hormonal activity in a mixture of EDCs is higher than the individual levels of each contaminant added together (Gilbert and Epel, 2009). Since there are no one-contaminant exposures to humans and other organisms, the synergetic effects are very relevant. Since the mechanisms of disruption are diverse and complex, a compound may be an agonist (mimicking the actions of a hormone) or antagonist (interfering or blocking actions of a hormone); it may alter transport of a hormone; or it may bind to more than one hormone receptor (Brown, 1994).

1.3 Endocrine Disruptors Dose Response

The effects on human and animal health may be even more difficult to assess due to the complexity of the endocrine system. The dose-responses appear to depend on the chemical structure and dose of the individual compound, the duration of the exposure, and the species, developmental stage (age) and gender of the organism (Metzler and Pfeiffer, 2001).

There are many reasons why it is not yet clear as to whether these contaminants are harmful. This is mainly because not all subgroups within a population react to the exposure in the same

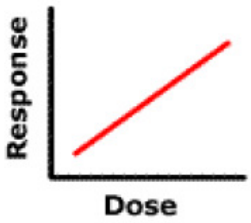
manner and the exposure effects may not be revealed until several generations. Because of the discrepancies in views and conclusions within current literature, there is no consensus suitable for the development of policy guidelines governing the presence of EDCs in the environment. Whether a decision is made to remove them from the water supply or treat for their presence is an ongoing debate in the drinking water community.

When pharmaceuticals and other drinking water contaminants are tested for exposure limits in a population, tests are typically conducted using healthy adults as a baseline guide. The estrogenic compounds and other hormonal steroids, however, have a greater disruption effect in a fetus and pubescent adolescences than mature adults. Numerous studies have shown that exposure to EDCs during critical periods of differentiation, at low environmentally relevant doses, can alter development programming resulting in obesity, diabetes and other adverse health effects. (Newbold, 2008; Baille-Hamilton, 2002). A new term, "environmental obesogens," has been created to describe these estrogenic contaminants and their adverse health effects (Grün and Blumberg, 2009).

When the endocrine system is subjected to a therapeutic, chronic dose of steroidal estrogens, it may produce decreased glucose tolerance, changes in menstrual cycle and breast changes (NLM, 2003). In men exposed to estrogens consistently, prolactinoma occurs, which is a growth of a noncancerous pituitary tumor that produces a hormone called prolactin. Prolaction is a hormone that triggers the breasts to produce milk, or lactate (Melmed *et al.*, 2008).

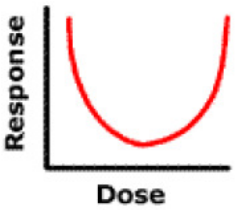
There is an increasing concern about the complexity in the biology of dose response curves. The traditional view holds that the effect is proportional to the cause. With this assumption, a dose response curve is either linear or monotonic. A linear dose response curve is produced when the dose and response have a direct relationship; thus, when the dose is increased the response is increased. A monotonic dose response curve shows that the direction of change does not reverse but there is no constant proportional relationship. In other words, nonlinearity says that the effect is not consistently proportional to the cause; rather it is an interacting "domino effect" (Masters, 1997). The monotonic curves can be described in mathematical terms to represent the response to each dose.

Non-monotonic dose response curves do not demonstrate a direct relationship between dose and response. In non-monotonic dose response curves, the shape of the curve reverses as the level of contamination increases. Simulation rather than mathematics is needed to determine a dose response for a contaminant that follows a non-monotonic curve. A non-monotonic curve is often referred to as a "U-curve," with high responses at low and at high levels of contamination. Inverted "U-curves" are also non-monotonic, but have the greatest response in intermediate ranges of contamination. The non-monotonic curves are also referred to as hermetic dose response curves (Baldi and Bucherelli, 2005; Calabrese, 2009; Mattson, 2008). Figure 1-2 shows pictorial descriptions of various dose response curves.



A. Linear Curve

B. Monotonic Curve



C. Non-Monotonic Curve

D. Non-Monotonic Curve

Figure 1-2: Dose Response Curves (adapted from Colburn, 1997)

Estradiol has been found in multiple dose response studies to follow a strong non-monotonic curve over a wide range of exposures (Christian and Gillies, 1999; Welshons *et al*., 2003). Welshons *et al*. concludes that within the range of concentrations of estradiol typically found in the environment (parts per quadrillion to parts per trillion), responses to estradiol are mediated by the estrogen receptor. At higher levels, those in the range normally used in toxicological experiments, the impact of estradiol is not receptor mediated. Figure 1-3 is a dose response curve for estradiol, the most common endogenous estrogenic compound.

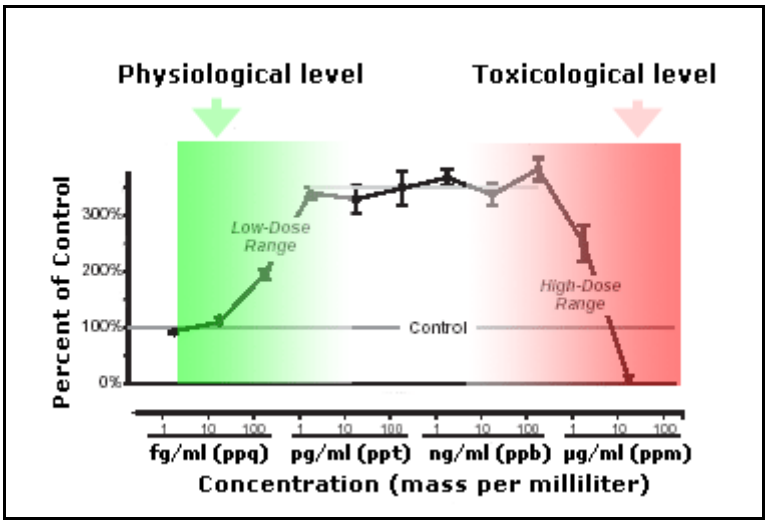


Figure 1-3: Estradiol Dose Response Curve (Welshons *et al.*, 2003)

Another classic example of non-monotonic dose-response curves comes from a study by vom Saal et al. examining the effect of the synthetic estrogen diethylstilbestrol (DES) on pregnant mice and their offspring (vom Saal *et al.*, 1997). This study measured the prostate weight in the male offspring of pregnant mice given a single dose in the range of 0 to 200 ng DES/g body weight. Figure 1-4 is a graph of the results of DES dose versus prostate weight.

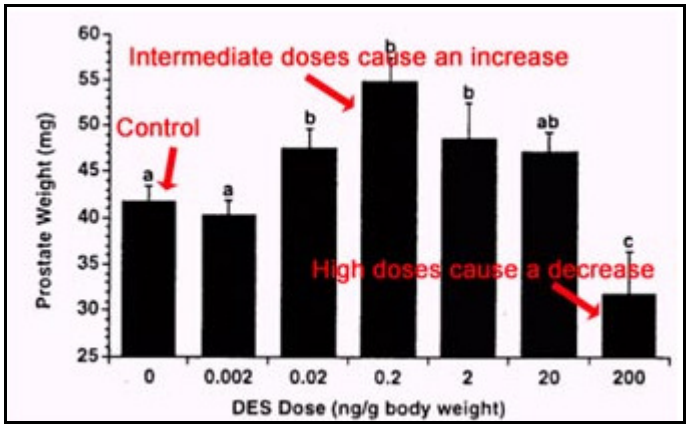


Figure 1-4: Dose Response of Pregnant Mice Given DES and Measured Response of Offspring Prostate Weight (vom Saal *et al.*, 1997)

According to vom Saal *et al.*, this type of inverted U function is typical of studies of the effects of estrogen on development. Due to both vom Saal and Welshons research findings, it must be considered that permanent alteration in the functioning of estrogen-responsive organs in animals and humans could occur due to exposure during fetal life. This exposure could be a result of low doses of estrogenic chemicals present in drugs (i.e. birth control pills) or from environmentally relevant concentrations of estrogenic chemicals present in food, water, and air (vom Saal, 1997).

The non-monotonic curves for EDCs could and should change the calculation to determine the no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) (Calabrese, 2009; DeSimone, 2009). Because of the non-monotonic dose response curves it is important not to rest on extrapolating high dose curves down to the low end of EDC exposure.

1.4 Steroidal Endocrine Disruptors

In the endocrine system, there are two major receptor molecules, ERα and ERβ, for the steroidal estrogenic hormones. The binding ligands are activated by 17β-estradiol or other estrogenic compounds that can dimerize and bind to the regulatory regions of estrogen-sensitive genes (Nelson and Cox, 2004). When the receptors bind with endogenous or exogenous estrogens, it activates a "signaling cascade" of cellular actions or binds to a DNA sequence (estrogen-responsive elements) and controls the transcription of the genes (Gilbert and Epel, 2009). Figure 1-5 shows the five estrogenic EDCs that are discussed in this research; including three natural estrogens (E1, E2 and E3) and two synthetic estrogens (EE2 and DES). The five EDCs are steroidal hormones, which are in the lipids class.

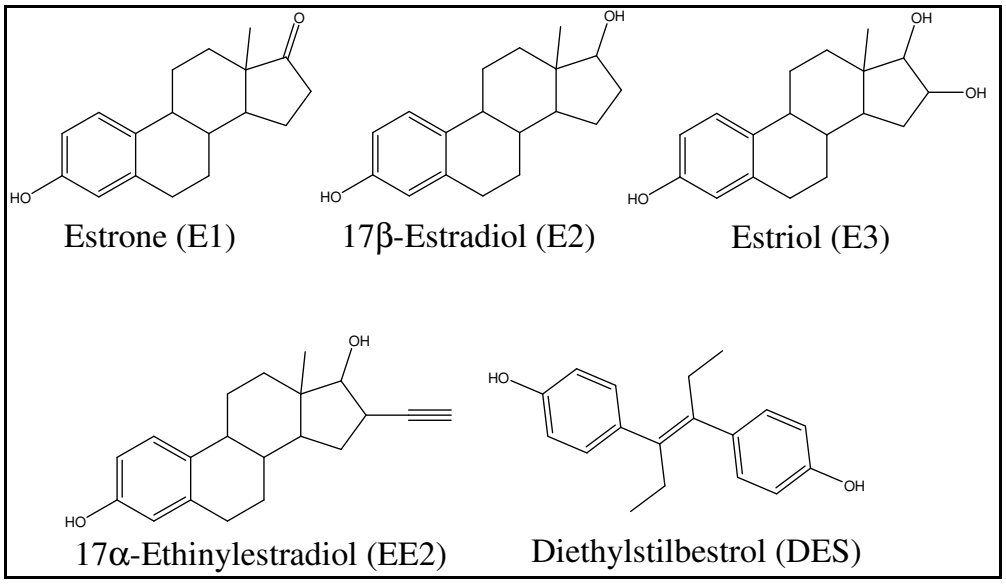


Figure 1-5: Estrogenic Endocrine Disruptors

Relative estrogenic strength of the five estrogens is as follows from highest to lowest biological activity: DES > EE2 >>E2 > E1> E3. The relative strengths are based on the source of the estrogenic compound (endogenous versus exogenous), the origin of the compound (natural versus synthetic), the abundance of the compound in the body naturally and the activity of the estrogen (McLachlan and Arnold, 1996). By considering the relative strengths, an estrogen equivalent factor effect can be found relative to estradiol or various bioassay responses (Campbell *et al.*, 2006).

The estrogenic drugs, such as ethinylestradiol, found in birth control pills are more stable and remain in the body longer than natural estrogens, like estradiol. Natural hormones are short-lived, do not accumulate in tissue and are easily degraded by our bodies. Most natural estrogens stay in the bloodstream only minutes or at most a few hours (Tait *et al.*, 1991). After that,

enzymes in the liver degraded the hormones into smaller compounds. These components are either flushed out as a waste product or reused to build other molecules.

In contrast to natural estrogens, estrogenic drugs and synthetic environmental estrogens are not easily or readily degraded; are long-lived, remaining intact in the environment and in living organisms for many years; and can accumulate in the natural world and within the fat and tissue of animals and humans. However, they are not nearly as persistent as pesticides and other environmental estrogens. Upon exposure, some of these estrogenic chemicals can be either flushed out or a portion can be absorbed into the body where they can bioaccumulate in fat and muscle (Cameron *et al.*, 1993; Mendel *et al.*, 1989; Miljoprojekt, 1995; Thomas *et al.*, 1992). Because most are lipophilic, they tend to accumulate in fatty tissue and stay there for years. During stress, such as pregnancy or breast feeding, these substances can be released from fat and redistributed or passed on to offspring. There is no doubt that humans are all exposed to some environmental estrogens. Whether or not the accumulated amounts are enough to produce long-term health problems is unknown.

Once steroid hormones are inside the body, they are metabolized primarily by conjunction with groups that interfere with interaction of the steroid to its binding proteins or receptors and render them more soluble in blood plasma (Norris and Carr, 2006). The majority of metabolism occurs in the liver; thus, the steroid hormones can then be readily excreted in sweat, urine and feces.

The biological pathway which produces the natural estrogens begins with cholesterol and is referred to as steroidogenesis.

Figure 1-6 shows the pathways from cholesterol to the production of the natural hormones including the estrogenic hormones; estrone, estradiol and estriol.

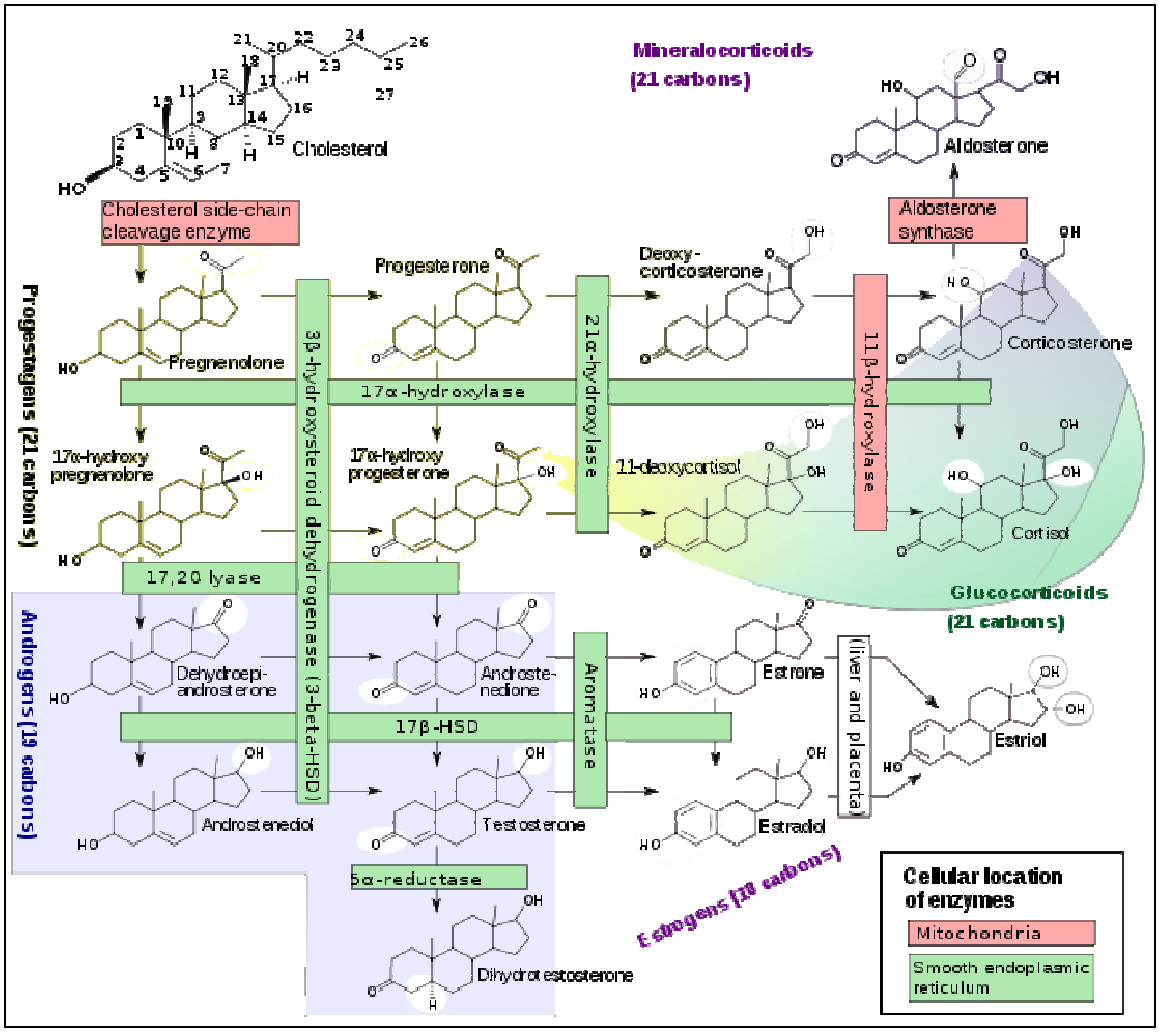


Figure 1-6: Steroidogenesis (Boron *et al.*, 2003)

The development of secondary sex characteristics in women is driven by estrogens, to be specific, estradiol. Estradiol, like other steroids, is derived from cholesterol. After side chain cleavage and utilizing the delta-5 pathway or the delta-4 pathway, androstenedione is the key intermediary. A small amount of the androstenedione is converted to testosterone, which then

undergoes conversion to estradiol by an enzyme called aromatase. In an alternative pathway, androstenedione is "aromatized" to estrone, which is subsequently converted to estradiol (Gilbert and Epel, 2009). Based on the steroidogensis diagram above, it is clear that these pathways are extremely important for normal body function and that there are multiple reactions that can be disrupted and/or magnified with “outside” contaminants, such as estrogenic environmental contaminants.

1.5 Environmental Estrogens

Both natural and synthetic estrogens have been detected in the environment. Even at the low levels found in the environment, these estrogens are still classified as endocrine disrupters due to their biological activity that is present at low concentrations. This research focuses on three natural estrogens (estrone, estradiol and estriol) and two synthetic estrogens (ethinylestradiol and diethylstilbestrol). The main emphasis is devoted to the synthetic estrogens due to their magnitude of biological activity, their longevity and their stability in comparison to the natural estrogens.

1.5.1 Estrone

Estrone (E1) is a naturally occurring estrogen that is produced in the ovaries and the adrenal glands. The chemical formula for estrone, shown in Figure 1-7, is a ketone $C_{18}H_{22}O_2$ (MW 270.37 g/mole). The solubility of estrone in water is 4.6 mg/L or 17 µM. The pKa of the phenolic moiety in E1 is 10.34 (Huwitz and Liu, 1977).

The IUPAC name, or International Union of Pure and Applied Chemistry nomenclature, for E1 is 3-hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16- decahydrocyclopenta[a]phenanthren- 17-one.

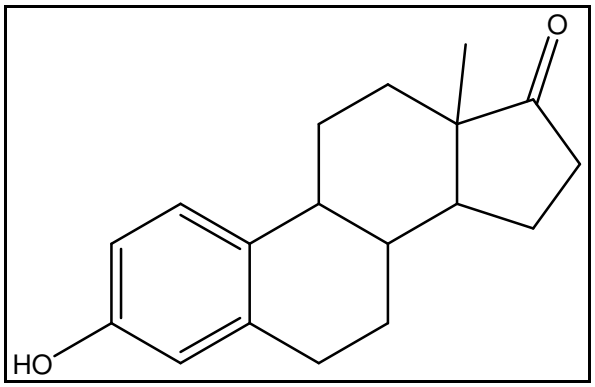


Figure 1-7: Estrone (E1)

In prepubescent children, men and postmenopausal women, a major quantity of estrone is derived from secondary tissue conversion of androstenedione, a steroid hormone. Estrone is a primary estrogenic component of several pharmaceutical formulations, including those containing conjugated and esterified estrogens (Petrovic *et al.*, 2004). Because of the extensive use of E1 in the treatment of estrogen deficiency and the incomplete metabolism by the users, significant concentrations have been observed in wastewater.

This endocrine disrupter is part of an emerging group of contaminants that have been noted for their presence in surface water (Thomas and Colburn, 1992). Some EDCs, similar to E1, have been linked to birth defects, infertility, immune system suppression, deformities to the reproductive organs, and various other health problems (Thomas and Colburn, 1992).

Estrone is the least abundant of the three natural hormones; estrone, estradiol and estriol. E1 is relevant to health and disease states because of its conversion to estrone sulfate, a long-lived derivative, which acts as a reservoir that can be converted as needed to the more active estradiol. Estrone sulfate is commonly found in wastewater, primarily from urine.

1.5.2 Estradiol

Estradiol (E2) is an endogenous, natural estrogen that is produced from a conversion of estrone to estradiol metabolized through the liver and excreted from the body through urine (Gore, 2007). This natural estrogen is the predominant sex hormone found in females and is typically assumed as the structure of estrogen; however, it is one of a group of estrogens. Within the female body, this hormone is responsible for changes in the body shape; affecting bones, joints and fat deposition. Estradiol is present almost always in the reproductive female body. Estradiol is also present in males but at much lower levels. E2 has a chemical formula of $C_{18}H_{24}O_2$ (MW 272.38 g/mole) with a chemical structure shown in Figure 1-8.

The IUPAC name for estradiol is (17β or 17α)-estra-1,3,5(10)-triene-3,17-diol. There are two forms of estradiol 17α and 17β, referring to the position of the hydroxyl group on C17. For the purposes of this research, the term estradiol will refer to 17β-estradiol, which is more commonly found in surface waters and drinking waters.

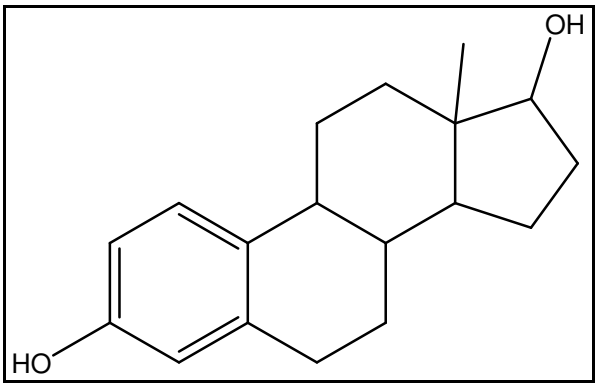


Figure 1-8: 17β-Estradiol (E2)

Like estrone, estradiol is almost insoluble in water, with a solubility limit of 3.600 mg/L (13.22 μM) at 27ºC (Yalkowsky, 1987). The pKa of phenolic moiety is 10.46 (Huwitz and Liu, 1977).

Estradiol is the most biologically active naturally-occurring estrogen. In addition to occurring naturally in the body, estradiol is also an active ingredient in many medications for birth control, hormone replacement therapy, infertility treatments and vaginal infections. These medications are delivered to the body through a variety of methods, including oral ingestion, trans-dermal application, injections and ointments. There are numerous adverse effects that are associated with an imbalance of estradiol. These effects range from minor health problems such as nausea, migraines and dizziness to major health problems such as breast cancer, strokes and heart attacks. E2 has also been linked to birth defects and deformities to the reproductive organs. A derivative form of estradiol is EE2.

1.5.3 Estriol

Estriol (E3) is also a natural estrogen like estrone and estradiol. Figure 1-9 shows the chemical structure of estriol, which has a formula of $C_{18}H_{24}O_3$ and a molecular weight of 288.38 g/mole. The pKa of the phenolic moiety in estriol is 10.38 (Huwitz and Liu, 1977). According to Campbell *et al.*, the solubility of estriol is 32 mg/L in water, which is significantly higher than the other steroidal estrogens (2006).

Estriol's IUPAC name is (8R,9S,13S,14S,16R,17R)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,16,17-triol.

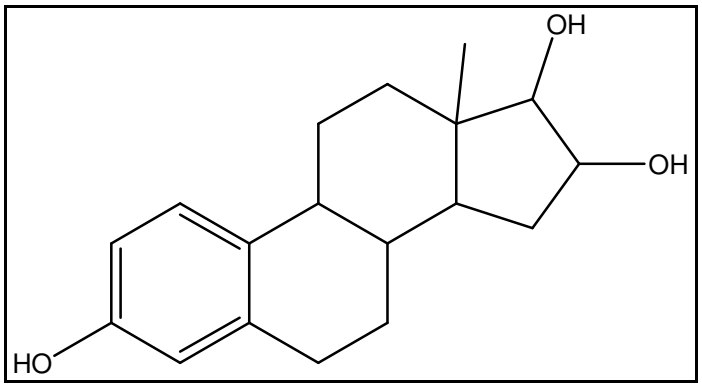


Figure 1-9: Estriol (E3)

Estriol is abundant primarily during pregnancy and is a common metabolite of estrone and estradiol in animals and humans. E3 is excreted in humans as conjugated and unconjugated 2-hydroxy estriol after hydroxylation (IARC, 1979). There is limited evidence in experimental animals for the carcinogenicity of estriol (IARC, 1999).

Research has shown that estriol in the presence of estradiol will act as an antiestrogen, therefore, reducing the activity of estradiol (Melamed, 1997). This research suggests that estriol assumes a protective role in opposing carcinogenic effects of estradiol.

1.5.4 Ethinylestradiol

Ethinylestradiol (EE2) is an exogenous, synthetic, steroidal estrogen which is commonly used as the active ingredient in birth control medications. EE2 has an IUPAC name of 19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol and is synthesized from estrone. Figure 1-10 shows the chemical structure of EE2, which is the same as estradiol except for the presence of the 17α-ethinyl group. The ethinyl substitution at the C17 position inhibits first-pass metabolism (absorption in the gut wall) of EE2, thereby decreasing the metabolism of the drug and increasing the biological half-life (INCHEM, 2005).

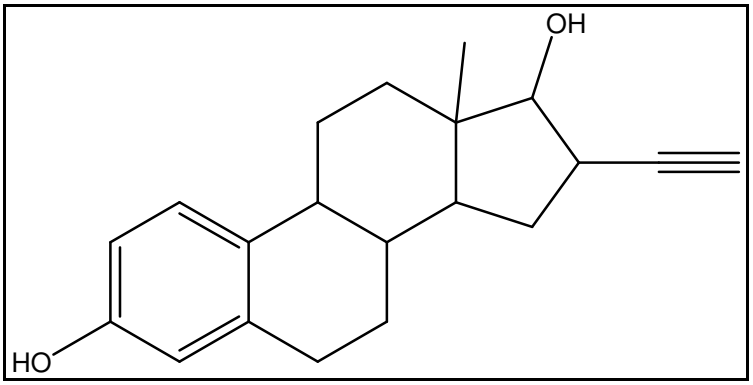


Figure 1-10: 17α-Ethinylestradiol (EE2)

The chemical formula for EE2 is $C_{20}H_{24}O_2$ with a molecular weight of 296.41 g/mol. EE2 is almost insoluble in water with a solubility of 4.8 mg/L (Campbell *et al.*, 2006). The pKa of EE2 phenolic moiety is 10.40 (Lee *et al.*, 2005).

EE2 is a major component of hormonal contraceptive devices. Combined forms of hormonal contraception contain EE2 and a progestin, which both contribute to the inhibition of Gonadotropin-releasing hormone (GnRH), Luteinizing-hormone (LH), and Follicle-stimulating hormone (FSH). These hormones are released by the pituitary and are involved in reproduction. The inhibition of these hormones accounts for the ability of these birth control methods to prevent ovulation and thus prevent pregnancy (Gore, 2007).

Like other steroidal hormones, EE2 is thought to act primarily through the regulation of gene expression. As a lipophilic hormone, it diffuses readily through cellular membranes to bind to estrogen receptors situated in the nucleus. The receptor interacts with a specialized nucleotide sequence, resulting in either an increase or decrease in the transcription of hormone regulated genes (INCHEM, 1997).

1.5.5 Diethylstilbestrol

Diethylstilbestrol (DES) is a synthetic non-steroidal estrogen (teratogen) which was used in growth hormone in the meat industry (1960s) and in birth control (1970s). Figure 1-11 shows the structure of DES, with two phenol groups connected by 4,4'-(3*E*)-hex-3-ene at C3 and C4. The IUPAC name for DES is 4,4'-(3*E*)-hex-3-ene-3,4-diyldiphenol.

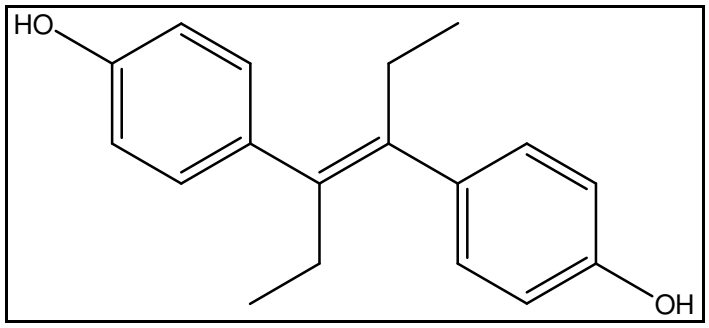


Figure 1-11: Diethylstilbestrol (DES)

Molecular weight of diethylstilbestrol is 268.35 g/mol with a chemical formula of $C_{18}H_{20}O_2$. The solubility of DES in water is 0.0956 mg/L at 25 ºC, which is almost insoluble (Syracuse Research Corporation, 1988). The pKa of the phenolic moieties in DES are 9.55 and 10.19 (Box et al., 2009).

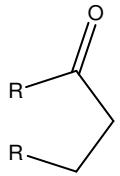
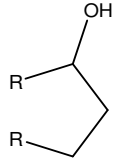
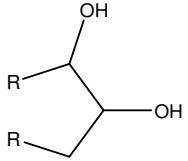
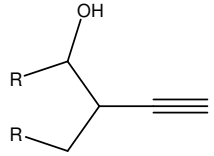
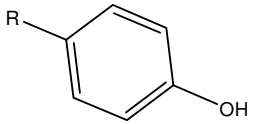
Diethylstilbestrol was the earliest synthetic form of the hormone estrogen. It was once widely prescribed to prevent miscarriages and premature births in the 1950s and 1960s. DES is still available for prescription in the US to treat breast cancer and prostatic cancer. Both treatment uses of DES are for only select patients with metastatic or advanced disease (MedTerms™ Medical Dictionary, 2002). DES is also currently used in canine and feline veterinary medicine for treatment of urinary incontinence, prevention of pregnancy, treatment of prostatic hypertrophy and treatment of tumors (Forney, 2004).

The side effects of DES are quite drastic. According to the CDC, DES usage is linked to vaginal cancer (clear cell adenocarcinoma, CCA) in the daughters (DES Daughters) resulting from a pregnancy that was prescribed DES; women prescribed DES while pregnant have increased risk

of breast cancer; and there is an increased risk for non-cancerous epididymal cysts in the sons (DES Sons) resulting from a pregnancy for a woman that was prescribed DES (2003).

Table 1-1 summarizes the characteristics of the environmental estrogens discussed in the previous sections.

Table 1-1: Summary of the Properties Associated with each Estrogenic Compound

Estrogen	Abbreviation	Origin	Chemical Formula	Molecular Weight (g/mol)	Differing Functional Group	Solubility in Water	pKa (s) of Phenolic Moiety
Estrone	E1	Endogenous	$C_{18}H_{22}O_2$	270.37	 Ketone on C17	4.6 mg/L (17 µM)	10.34
Estradiol	E2	Endogenous	$C_{18}H_{24}O_2$	272.38	 Hydroxyl group on C17	3.6 mg/L (13.2 µM)	10.46
Estriol	E3	Endogenous	$C_{18}H_{24}O_3$	288.38	 Hydroxyl groups on C16 and C17	32 mg/L (110 µM)	10.38
Ethinylestradiol	EE2	Exogenous	$C_{20}H_{24}O_2$	296.41	 Hydroxyl group on C17 and Ethinyl group on C16	4.8 mg/L (16 µM)	10.40
Diethylstilbestrol	DES	Exogenous	$C_{18}H_{20}O_2$	268.35	 Additional Phenolic group	0.096 mg/L (0.36 µM)	9.55 and 10.19

1.6 EDC Drinking Water Contamination

The following sections address the multiple sources that can contribute to contamination, the fate of the contaminants and the implications for these environmental contaminants in the drinking water supply.

1.6.1 Source of EDCs in the Environment

There is increasing concern that wastewater effluent discharge streams located in close proximity to drinking water intakes may compromise drinking water supplies. The resulting increased contaminant loads may place a heavy burden on drinking water utilities (van der Linden, 2008).

There are many pathways for contaminants to enter the drinking water supply, by natural and/or anthropogenic means. Contamination of water supplies can be either from point or non-point sources. The estrogens studied in this project are both synthetic and natural, which suggests multiple sources for contamination.

Since many emerging contaminants are found in discharge streams from wastewater treatment plants, there is an increasing need to monitor and evaluate the distance between the waste stream and the drinking water source associated with surface waters. Many of these emerging contaminants have not been measured for surface waters in the United States because the analytical technology needed to detect trace levels is quite costly. Now that there is technology available to measure at trace levels with decreased cost, new strategies are needed to help monitor the quality of our source waters used for municipal drinking water. If improved strategies are set in place to identify impaired areas, then water monitoring samples will only be

needed in select places, thereby, decreasing the number of samples needed to monitor a watershed.

A major concern within the emerging contaminant groups has been the pharmaceuticals and personal care products (PPCPs) that have entered the environment. Many of these compounds have been found in selected areas around the county, with most detections stemming from wastewater discharge (Servos, 2005). Since many wastewater treatment plants discharge directly in surface waters that are upstream of drinking water intakes, it is very important to understand the persistence of PPCPs in the aquatic environment.

Estrogenic EDCs can be introduced to the environment from wastewater, pharmaceutical industries, hospitals and animal farming (confined animal feeding operations or CAFOs), etc. One of the main sources of the natural water pollution by EDCs is sewage-treatment plants (STP). It has been reported that, because of partial removal or formation of an active form during the process of sewage treatment, endocrine disrupters are released in surface waters or adsorbed onto sewage sludges (Gomez *et al.*, 2006; Ternes *et al.*, 1991). Drinking water is also contaminated by these chemicals and their breakdown products found in industrial discharge as well as sewage effluent. Figure 1-12 shows a schematic of several sources and sinks associated with EDCs.

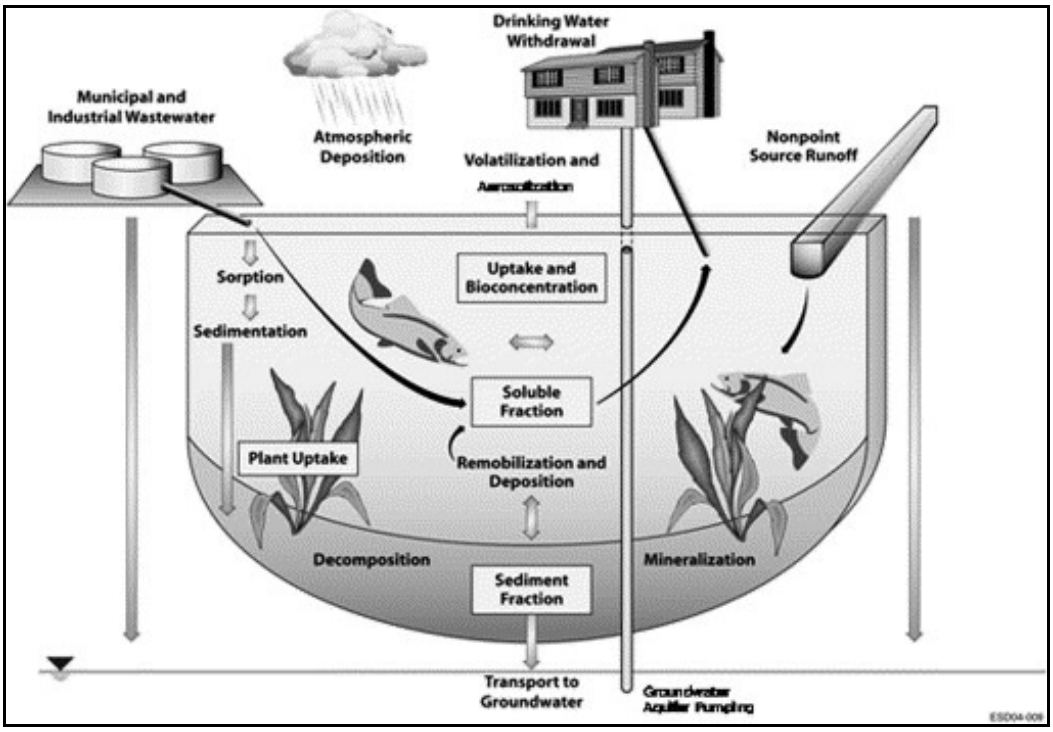


Figure 1-12: Fate of EDCs in the Environment (Campbell *et al.*, 2006)

The main source of estrogens in municipal wastewater is urine, which contains 67-80% of estrogens excreted daily (Maurer *et al.*, 2006). The mean of the per capita loading for influent concentrations that were determined for the model are as follows: 3.3 µg of E2/person/d, 13.8 µg of E1/person/d, 0.89 µg of EE2/person/d. These concentrations were found by dividing the flow by influent concentrations by the average excretion of the respective estrogens (Johnson *et al.*, 2004).

The occurrence level of EDCs in drinking water primarily depends on the level of wastewater contamination within a watershed (AwwaRF, 2008). Because varying levels of EDCs are used/disposed within watersheds, the total amount of biologically active contaminants introduced to the environment has a wide range depending on the removal rates of the wastewater treatment

systems. According to an AwwaRF publication on the occurrence on EDCs, steroid hormones have a detection frequency in wastewater effluents of over 60 % in both North America and Europe, with median concentrations of >12 ng/L (2008). Even if the removal efficiency of steroidal EDCs in wastewater treatment is 90% (Snyder *et al.*, 2003), then 1.2 ng/L of the steroid hormones remains in the effluent. Assuming a dilution ratio of wastewater effluent to surface waters of 1:1000, the absence of degradation between treatment plants and a drinking water treatment removal efficiency of 90% (Snyder *et al.*, 2003), then 0.12 pg/L of the steroid hormones may be introduced into the drinking water distribution system.

Figure 1-13 shows the percent EE2 metabolized and excreted by a female taking birth control where the active ingredient is EE2.

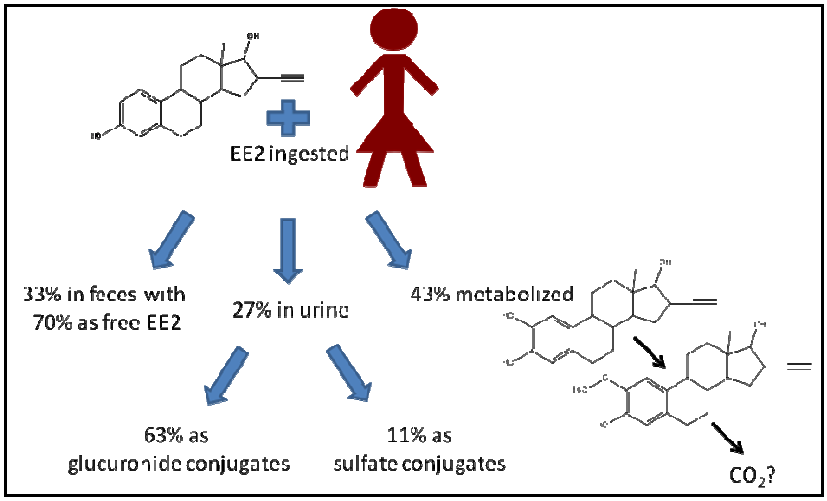


Figure 1-13: Excretion of Ethinylestradiol in the Body (adapted from Johnson *et al.*, 2004)

The conjugates of estrogenic EDCs found in wastewater have been shown to come from urine. These conjugates will deconjugate within the collection systems and the treatment plants. The

organic loading has been suggested as the key parameter for degradation of estrogens due to the potential for competitive substrate inhibition limiting biodegradation. The removal efficiencies for steroid estrogens in wastewater treated with nitrification/denitrification processes are greater than 90 %; however, there is a small amount of EDCs remaining in the treatment plant effluent which is then released back to the environment (Koh *et al.*, 2009).

Synthetic estrogens can also be introduced to wastewater when unused birth control pills and other pharmaceuticals are flushed for disposal. After synthesis, DES can be introduced to the environment during transport, storage or disposal. If released to soil, diethylstilbestrol is predicted to strongly adsorb to the soil. If released to water, diethylstilbestrol may bioconcentrate in aquatic organisms and strongly adsorb to suspended solids (Spectrum Laboratories, 2003).

There are numerous surface waters that could be categorized as compromised because of water reuse, or source waters containing a substantial percentage of wastewater effluent. Even with the ability of current technology to detect contaminants, the process is very labor intensive and very expensive. There are many discharges, other than sewage, that can release low levels of PPCPs into the environment. Once the water system is compromised by decreased source quality, the costs to produce high quality water rapidly escalate.

The water intake and wastewater discharge data can be obtained from the EPA's list of National Pollutant Discharge Elimination System (NPDES) permits for Massachusetts. The permit provides two levels of control with technology-based limits and water quality-based limits. The

technology-based limits determine if there is sufficient analytical ability to provide protection of the water body. The second level of the NPDES permits, water quality limits, is based on the ability of dischargers in the same industrial Office of Wastewater Management-Water Permitting category to treat wastewater (EPA, 2008). The NPDES Database for the Commonwealth of Massachusetts is available through the EPA; however, the current permits do not include PPCPs or biologically active compounds.

Figure 1-14 and Figure 1-15 show the drinking water intake sites and the NPDES Permit sites for the Commonwealth of Massachusetts, respectively.

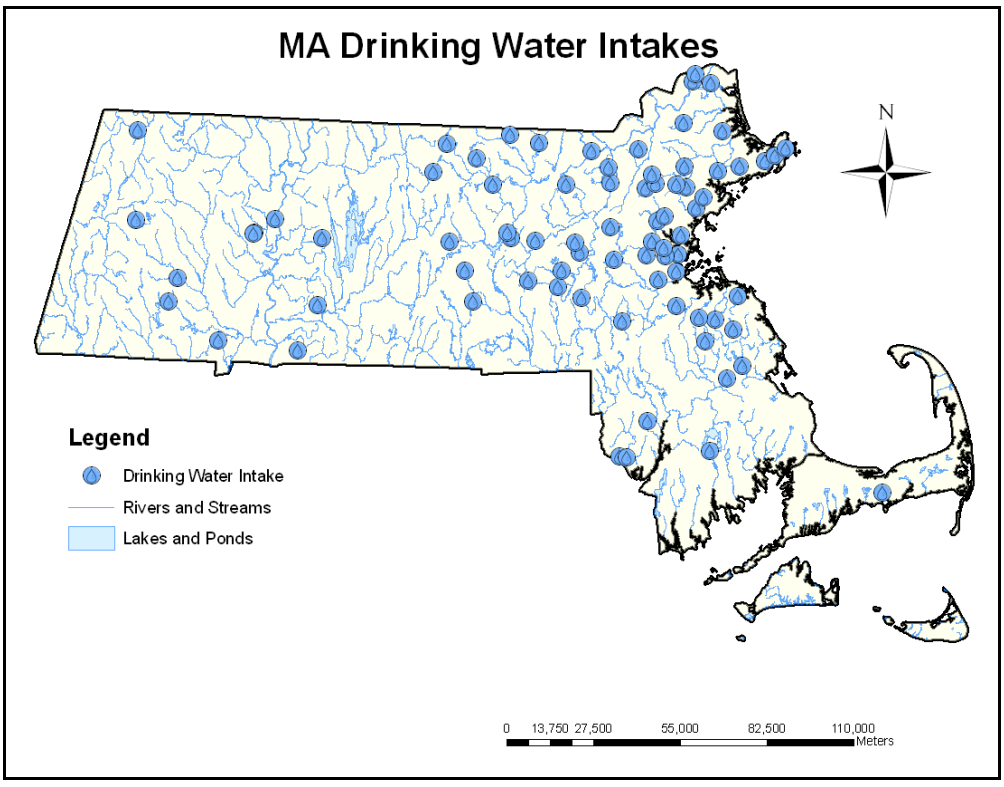


Figure 1-14: Massachusetts Drinking Water Intake Sites

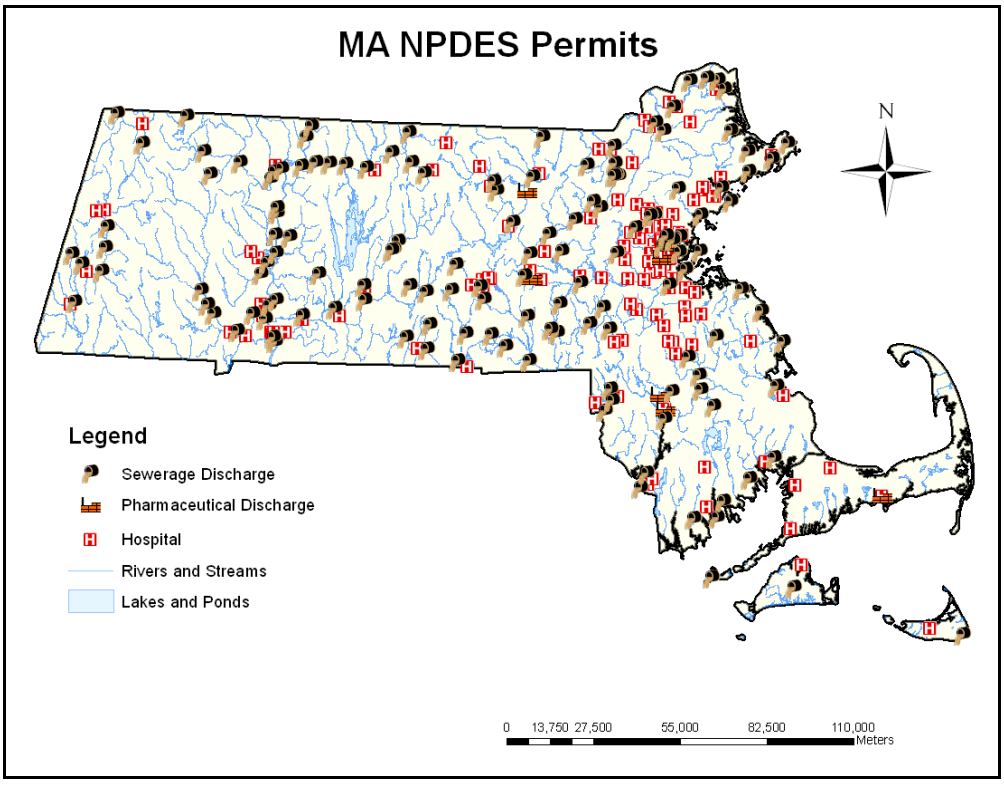


Figure 1-15: Massachusetts NPDES Permit Sites

Proximity to potential sources of steroidal estrogen contamination can create "High Priority Sampling Zones" which would direct researchers to high risk drinking water sources. Sites can then be either eliminated or included in frequent monitoring for estrogenic contamination.

There are mounting problems with monitoring and managing these forms of environmental pollution. Environmental management of estrogenic EDCs will rely on source reduction, limiting exposure to vulnerable populations, and treatment and remediation of waste streams or contaminated sites. Large scale monitoring networks are needed to accurately understand and model the transport mechanisms of these contaminants.

The United States Geological Survey (USGS) has implemented the National Water Quality Assessment Program, which has been monitoring estrogenic compounds in wastewaters, groundwaters and surface waters for the last decade (Barnes *et al*., 2002; Berdanier and Clay, 2011; DeSimone, 2009). Even with these USGS studies, more research needs to be started on eliminating or inhibiting the contaminant sources to help mitigate the increasing levels of EDCs being found in raw drinking water.

1.6.2 Fate of EDCs in the Environment

Once endocrine disruptors are introduced to the environment many possible scenarios can occur. The EDCs can remain in the vicinity of the initial discharge or they can be transported down-gradient. When EDCs are discharged to soils, the compounds can either be adsorbed to the soil particles or volatilized into the atmosphere or transported in the dissolved state by percolation, groundwater flow or overland runoff. Degradation could occur depending on the chemical components of the contaminant and the chemical surroundings. Since the volatization rates are low for most estrogenic EDCs and their atmospheric half lives are on the order of minutes, exposure to these compounds is only significant for those who handle them daily or if a large contamination occurs (SRC, 1988).

If the contaminants are transported or introduced into a water source, either ground or surface waters, then significant EDC contaminant exposure to the general population is most likely through drinking water. Even though the solubility (Table 1-1) of the estrogenic EDCs is relatively low, the concentrations found in the environment are within the physiological response level to the low dose, as discussed in Section 1.3 *Endocrine Disruptors Dose Response.*

The extent of estrogenic EDC biodegradation in soils and natural waters is not known, although the synthetic estrogens have been shown to be resistant to degradation in activated sludge (Gomes *et al.*, 2009). Based on the influent and effluent concentrations in the study done by Johnson *et al.*, there are no relationships that can be attributed to removal by specific steps within activated sludge treatment (2004). If released to surface or ground waters, synthetic estrogens and natural estrogens can bioconcentrate in aquatic organisms and strongly adsorb to suspend solids and sediments (Langston, 2010).

There have been multiple studies that have found these estrogenic contaminants in drinking water (Barnes *et al.*, 2002; Kolpin *et al.*, 2002; Lévi *et al.*, 2002; Vethaak *et al.*, 2002). With the increase in analytical detection potential, more and more surface and ground waters will prove to be contaminated with EDCs. The levels of detection are usually measured in parts per trillion (ppt) or parts per quadrillion (ppq). Table 1-1 presents the mean contamination levels in wastewater, surface water and drinking water for estrogenic compounds.

Table 1-2: Mean Levels of Estrogenic Contamination

EDC Contaminant	Levels in Wastewater Influent	Levels in Surface Water	Levels in Drinking Water
Estrone	20-30 ng/L[*]	0.1-4 ng/L[#]	0.2-0.6 ng/L[#]
Estradiol	10-15 ng/L[*]	0.15-3.6 ng/L[#]	0.2-2.1 ng/L[#]
Estriol	60-80 ng/L[*]	1.0-2.5 ng/L[1]	0.03 ng/L[3]
Ethinylestradiol	1-5 ng/L[^]	0.1-5.1 ng/L[#]	0.15-0.50 ng/L[#]
Diethylstilbestrol	4.8–12.4 ng/L[4]	2 ng/L[2]	0.11-0.26 ng/L[+]

[*]Hashimoto *et al.*, 2007; [^] Joss *et al.*, 2004; [#]Kuch and Ballschmiter, 2001; [1]Cargouët *et al.*, 2004; [+]Rurainski *et al.*, 1977; [2]Henriques *et al.*, 2010; [3]Wise *et al.* 2011; [4]Jin *et al.*, 2008

Even though these concentrations are low compared to other sources of EDCs that individuals encounter daily, it is still cause for concern that a mixture of highly physiologically active contaminants are included in drinking water and that the overall effect has not been determined conclusively.

1.6.3 Implications of EDCs in the Environment

There are many opinions as to whether or not the presence of endocrine disruptors in the environment causes any effects to an individual. One view suggests that the minute amounts of these contaminants do not have any effect when compared to the overall concentrations of estrogenic substances that are consumed daily (Benotti *et al*, 2009; Westerhoff *et al.*, 2005; Wise *et al.*, 2011). Yet another interpretation proposes that it is the complex mixture of many different EDCs and not the actual concentration which is harmful (Matthiessen, 2000). Yet another interpretation of endocrine disruptors in the environment is that effects are more severe during developmental stages and that overall effects will not become apparent until future generations (Welshons *et al*., 2003; vom Saal, 1997). The research that has been conducted to date has not provided a definitive answer as to which view is correct.

This is an area where research design limits the possible conclusions. Researchers have been focusing on different population groups which have provided different dose-response outcomes. For instance, if an adult is exposed to EDCs, then the effects will be minimal, if any, compared to prenatal or pre-puberty exposure. Synergistic effects also impact the response to estrogenic contamination by either enhancing the biological activity of the mixture or decreasing the

biological activity of the mixture. This change in activity is directly related to the combination of antagonists and agonists present in the mixture of contaminants.

Environmental impacts have been observed in aquatic organisms and laboratory tests show animals responding negatively to exposures of EDCs. Many have postulated that responses to environmental EDCs are manifest in the general population, such as low sperm count in males, increased infertility, increased obesity, increased diabetes and numerous other general population phenomenons. These responses are discussed in Section 1.2 *Exposure to Endocrine Disruptors*.

The Associated Press published articles on pharmaceuticals and personal care products in drinking water in the spring of 2008 (NPR, 2008; Donn *et al.*, 2008). The public became alarmed about this report, partly due to their concepts about drinking water treatment and also due to the manner in which the information was released. Researchers have known of this contamination since the 1970s; however, the detection ability has greatly increased since that time (Rurainski *et al.*, 1977). The increasing amount of detection of EDCs is directly related to analytical power used to identify and quantify them; however, with the increase of surface and ground water contamination, drinking water will inevitably have an increase in contaminant load regardless of analytical ability.

Since the consequences of having low quantities of EDCs in water are not completely known, it is imperative that changes to wastewater and drinking water treatment techniques be considered in order to optimize degradation, while keeping the original integrity of the treatment system. Without placing adequate attention on EDCs in the environment, we may reach a tipping point

where increased hormonal activity could do irreparable damage to our ecosystems and future generations.

1.7 Conclusions

The research reviewed in this introduction has provided a basis to warrant increased concern of estrogenic endocrine disruptor in the environment. Along with reconceptualized dose-response curves and new findings in developmental biology (epigenetics), these latest interpretations have created a new understanding of the environmental health concerns associated with estrogenic compounds in drinking water.

To the average healthy adult, estrogenic EDCs in drinking water may have little effect on the endocrine system. To prepubescent children, however, the likelihood of adverse impacts increases due to the multiple biological mechanisms that are changing the body. The biggest impact and most immediate concern from EDCs are the vulnerability of embryos and fetuses, because their rapid development is dependent on the endocrine system.

With these issues in mind, the current research within the drinking water community needs to focus on the detection and quantification of these contaminants as well as identifying any byproducts associated with drinking water treatment to discern if more harmful contaminants are produced. There are no existing methods that are universally used for estrogenic EDC detection and quantification in drinking water and the treatment byproducts have not been identified for all of the contaminants of concern. Therefore, continuing research is needed to help in understanding the complete consequences of estrogenic endocrine disruptors in drinking water.

CHAPTER 2 Analytical Detection of EDCs in Drinking Water

2 Analytical Methods

2.1 Detection of EDCs in Drinking Water

Due to the chemical diversity of endocrine disrupting compounds, the range of instrumental techniques available for their analysis is very large. Within modern analytical techniques applicable to trace analysis of endocrine disrupting compounds (EDCs), gas chromatography (GC) and liquid chromatography (LC) in tandem with mass spectroscopy (MS) or with tandem mass spectroscopy (MS-MS), play an important role in providing sufficient selectivity and inherent sensitivity in the analysis of complex environmental matrices (Petrovic *et al.*, 2004). Most of the analytical procedures developed for environmental determination of EDCs and emerging contaminants have been designed for analysis of specific classes of compounds. Analysis of each byproduct of individual EDCs will assist in developing multi-residue methods in which different compound classes can be identified in a single analysis.

Driven by the estrogenic potency of these compounds and low environmental concentrations, the detection limits required for monitoring of EDCs are being pushed from microgram per liter (μg/L) to the nanogram per liter (ng/L) range and even to the picogram per liter (pg/L) range. The two most widely used forms of analysis, which can be used to accurately measure at these environmental concentrations, are GC-MS and LC-MS. Gas chromatography-mass spectroscopy has not been customarily used in the analysis of EDCs because the LC-MS-MS analysis of estrogenic compounds is much faster and less affected by error (Petrovic *et al.*, 2004). Because the GC utilizes the gas phases during analysis, a derivitization step must be added during sample preparation to condition analytes from the liquid to gas phase change.

Liquid chromatography separates analytes based on their solubility in the mobile phase and their affinity to the stationary phase, or column. Gas chromatography works on the same principles as the LC but uses a carrier gas phase instead of a mobile liquid phase. This research utilized two LC-MS-MS systems to produce an identification and quantification method for estrogenic endocrine disruptors.

The overall research goal using these analytical methods was to determine a suitable LC/MS/MS method for identification and quantification of estrogenic endocrine disruptors and their treatment byproducts. To achieve this goal, two existing methods were compared and a new method was developed. Once the treatment byproducts are identified, the method can be simplified or modified for less sensitive instruments to determine the EDC removal and by-product presence. It is anticipated that such degradation and detection techniques, supplementing the present drinking water purification procedures, will help to identify and prevent threats to water resources.

2.2 Analytical Methods Background

Due to the analytical challenges posed by low levels of EDCs in drinking water, new techniques for the detection and quantification of estrogenic compounds have been the subject of intense investigation. From the wide variety of EDCs, these analytical methods focused on estrogenic EDCs, including estrone (Sigma-Aldrich, >99% purity), estriol (MP Biomedicals, >97% purity), β-estradiol (MP Biomedicals, >98% purity), 17β-ethynylestradiol (Sigma-Aldrich, >98% HPLC grade purity) and diethylstilbestrol (Sigma-Aldrich, 99% purity).

There are many protocols for measuring organic contaminants at trace levels in water, and most call for some pre-concentration, separation and cleanup. SPE is one of the most widely used methods for pretreatment of these samples. Because of the slow and laborious nature of most SPE protocols, there is great interest in ways of reducing time and cost in EDC analysis. Several instruments which integrate automated SPE with LC/MS have been proposed for addressing these needs.

This section discusses the differences in a manual method and an innovative approach using an automated SPE-LC-MS-MS for trace analysis of EDCs. For this research the automated on-line system was the Waters AquaAnalysis System supplemented with a newly developed method. The manual off-line system used a Varian Elut SPS 24 extraction manifold with Dryfast Vacuum Pump and Waters Alliance 2695 Separation Module coupled to a Waters Quattro *micro*™ API Quadrupole Mass Spectrometer. The manual method was based on *EPA 1694 Method: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment and Biosolids by HPLC/MS/MS*, which is an accepted method for measuring PPCPs; this method, however, does not include estrogenic EDCs (2007). Both methods used the same type of LC separation column (Waters C-18 Atlantis® T3, 3µm 2.1x50mm Column) and the same mass spectrometer. The solid phase used in the SPE was the Waters Oasis® Hydrophilic-Lipophilic Balance (HLB) 60 µm extraction cartridges for both methods. The HLB cartridge contains a resin made from a co-polymer of divinybenzene and vinyl pyrrolidinone, which acts as an imbedded hydrophilic group and enhances retention of analytes (Young *et al.*, 1999). Thus, the only features compared

between methods were the quality and efficiency of sample preparation and sample introduction to the analytical instrumentation.

The performance of sample preparation and introduction was compared using the coefficients of determination, relative standard deviations and signal-to-noise ratios. The coefficient of determination (R^2) is the proportion of variability within a sample set using a linear regression model (Sen *et al.*, 1990).

$$R^2 = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2} \qquad \textbf{Equation 2-1}$$

Both low concentration and high concentration calibration curves were used to find a coefficient of determination. The low concentration included 1.0 ppt to 100 ppt and the high concentration included 25 ppb to 100 ppb.

The signal-to-noise ratio (S/N) is the ratio of the peak height (from a given mass load) to baseline noise (Young *et al.*, 1999). The minimum quantfication limits (MQLs) were determined based on the lowest concentration where chromatographic peaks had a signal-to-noise ratio of 10:1 or higher. The minimum detection limits were selected based on the lowest level at which an analyte could be detected, which was defined as a S/N of 2:1 or 3:1.

The relative standard deviation is the absolute value of the coefficient of variation (Sen *et al.*, 1990). This measurement was used to compare the instrument variability in different injections of the same sample and was expressed as a percent.

$$RSD\% = \frac{\sqrt{\sum_i \frac{(x_i - \bar{x})^2}{N-1}}}{\bar{x}} \times 100 \qquad \textbf{Equation 2-2}$$

The following sections further discuss and expand on the benefits and limitations of the off-line, manual methods and the on-line, automated method. This comparison was necessary because of the lack of standard methods and the enormous cost and effort required in measuring and monitoring EDCs.

2.3 Soild Phase Extraction Introduction

Solid-phase extraction (SPE) is an established technique that is routinely used for the extraction/concentration of target compounds and for removing interference from matrix components prior to chromatographic analysis (Alda *et al.*, 2003; Richardson and Ternes, 2005; Wells *et al.*, 1987). This sample pretreatment method is used to enhance the analytic signal to quantitatively analyze contaminants with liquid chromatography/mass spectroscopy.

SPE is commonly used for extraction of EDCs from environmental matrixes and operates on the principles of solubility and solid phase affinity of each analyte. The process includes the removal of organic contaminants from water and adsorbing them onto a solid phase followed by extraction and elution to concentrate the samples. Pre-extracted sample sizes typically range

from 500 mL to 1 L, with a final sample volume of 1 mL. The pre-sample size is based on the cost and practicality of the sample volume and the enhancement factor desired in the extracted sample. The solid phase employed in the extraction can vary widely based on the structure and composition of the analytes and can be contained in either cartridges or barrels depending on whether the system is off-line or on-line. Figure 2-2 shows the SPE steps when using a cartridge for the off-line method. The same procedures are used for on-line SPE systems; however, software programs control the loading, washing and extraction instead of the operator.

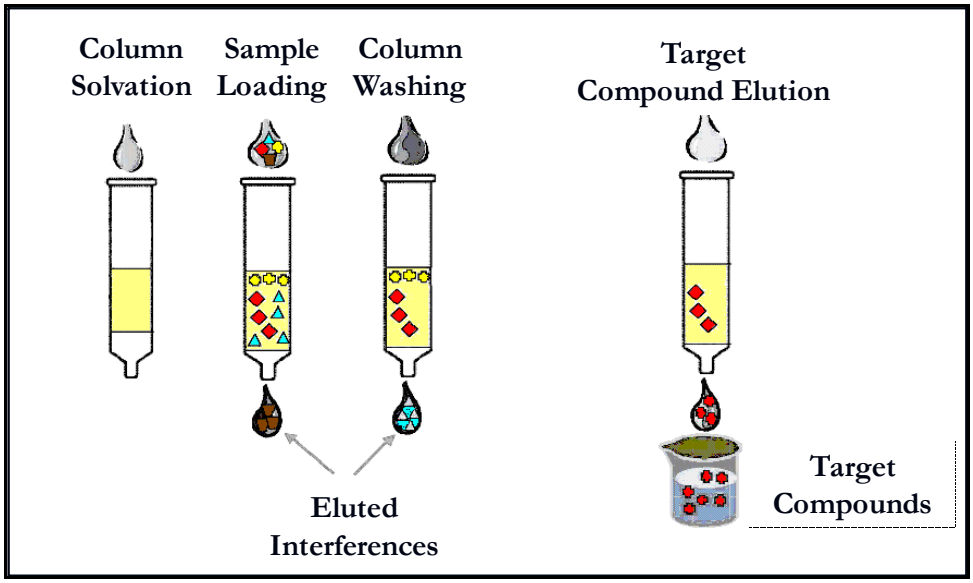


Figure 2-1: Solid Phase Extraction Using Columns
(adapted and modified from www.biotage.com)

Column solvation refers to the conditioning of the column to prepare the solid phase for sample loading. Depending on sample size, the sample loading step can require a considerable time to complete. With increased sample size, there is an increase in final concentration; therefore, sample extractions tend to result in a 1000 fold reduction in sample size and 1000 fold increase in analyte concentration, presuming complete recovery. Column washing prior to elution is an

extremely critical step used to decrease interference in MS analysis of target compounds. If this step is ignored, then the interfering compounds are also increased along with the analyte, causing increased signal-to-noise, false elevated signal intensity during analysis, or charge suppression in LC/MS.

There are many types of SPE available in the laboratory. The off-line manual extraction uses a vacuum manifold to draw the raw sample through SPE cartridges and manually administer column washing solvents and the elution solvents. After the 1 mL extracted sample is collected, a fraction of that sample is separated and analyzed using LC-MS. This SPE method is extremely laborious and there are many points within the numerous steps where error can be introduced.

For automated SPE devices, there are two forms available; automated off-line SPE excluding a detection system and automated online SPE including a detection system. The off-line SPE, excluding the detection system, uses software to control pumps and solvents to perform the same extraction as the manual method, with the raw sample being reduced to the 1 mL extracted sample. A fraction of this extracted sample is then injected into the detection system to be separated and analyzed. The on-line SPE system with a detection system also uses software to control solvents and samples introduced to the SPE columns, which are usually barrels made with the same solid phase as the cartridges. The “extracted” sample is then eluted off the barrel and the entire sample is separated and analyzed using LC-MS. Both of the automated SPE methods reduce operator error; however, the on-line version has further benefits in the increased size of the sample used in the analysis.

2.4 Molar Absorption Coefficients

2.4.1 Introduction to Molar Absorption Coefficients

Molar absorption coefficients can be used to verify concentrations of analytes measured by LC/MS/MS when the absorbance chromatograms are isolated from the total ion chromatogram. Molar absorption coefficients, $\varepsilon(\lambda)$, are determined by measuring the absorbance of pure solutions at various known concentrations in a 1 cm path length cuvette and performing a linear regression of the absorbance at each wavelength versus concentration according to the Beer-Lambert Law shown in Equation 2-3 (Schwarzenbach *et al.*, 2003).

$$A(\lambda) = \varepsilon(\lambda) b [EDC] \qquad \textbf{Equation 2-3}$$

Determining the molar absorption coefficients allowed solution concentrations to be validated by taking the UV absorbance of the EDC solution at the wavelength that corresponds to the local maximum calculated coefficient.

2.4.2 Molar Absorption Coefficients Methods

Since the estrogenic EDCs being studied have a multiple ring structure, their absorbance spectra have multiple local maxima and they have a unique curve associated with phenols. An Agilent 8453 UV spectrophotometer was used to measure the UV absorbance at wavelengths from 190 nm to 400 nm. The molar absorption coefficient curves were determined using a linear regression function, utilizing a range (1 to 10 mg/L) of known concentrations of each EDC in deionized water and their corresponding absorbance spectra. To produce an exact, known

concentration, the individual EDCs were first dissolved in methanol at 1.00 mg/mL concentrations and then serially diluted in water to the appropriate concentrations. It was assumed that after the dilutions, the methanol concentration was negligible. The corresponding molar absorption coefficient curves are shown in Figure 2-2.

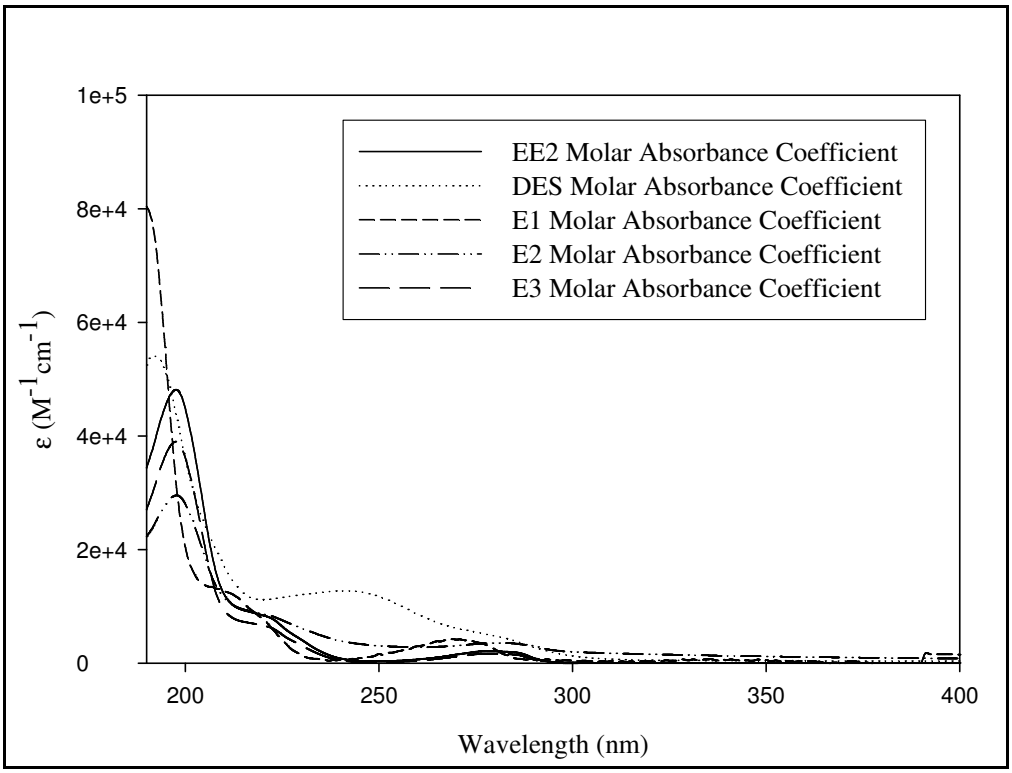


Figure 2-2: Estrogenic EDCs Molar Absorbance Spectra

The molar absorbance curves in Figure 2-2 follow similar patterns of two local maxima at 220 nm and 280 nm, which is due to the multiple ring structure for the estrogenic compounds. DES is the exception with only one local maxima at 240 nm because both rings are phenols and have similar vibration and rotation states due to the symmetry of the compound. Table 2-1 provides the maximum molar absorption coefficients and the corresponding wavelengths for each analyte.

Table 2-1: Molar Absorption Coefficients for Estrogenic EDCs

	Wavelength, λ (nm)	**Molar Absorption Coefficient, ε ($M^{-1}cm^{-1}$)***
Estrone	220 280	12,738 4,173
β-Estradiol	220 280	8,676 3,563
Estriol	220 280	6,636 1,680
17β-Ethinylestradiol	220 280	8,430 2,131
Diethylstilbestrol	240	12,753

*Values agree with Mazellier *et al.* for EE2 (2008), Puma *et al.* for E3 (2010), *Merck Index* for DES (2006). Absorption coefficients for E1 and E2 agree with Hurwitz and Liu, 1977; however, they do not agree with Puma *et al.* (2010).

2.4.3 Molar Absorption Coefficient Applications

For E1, E2, E3 and EE2, the molar absorbance intensities vary due to the differing functional groups on the C17. The differing moities have small effects on the overall spectra, which are dominated by the multiple ring structure. Because of these characteristic absorbance curves, when the ring is broken during a chemical reaction, the absorbance spectra for the byproducts are noticeably different from the parent compound. The differences between the parent and byproduct absorbance spectra could connote changes in functional groups or alterations in ring structures.

2.5 AquaAnalysis Method

2.5.1 AquaAnalysis Method Background

The Waters AquaAnalysis is a parallel on-line sample prep plus separation and detection system. The extraction portion of the on-line system implements reusable Oasis® HLB barrels (>4000 injections) (Mallet, 2008). The SPE and analytical instrument processing method had an injection volume of 5 mL and a combined run time of 20 minutes. Larger amounts of sample can be introduced to the system; however, a 5 mL sample size was chosen based on the extracted sample size reduction used in the manual SPE method, which is discussed *Section 2.6 EPA 1694 Method*. The typical sample vial for the AquaAnalysis was 20 mL in volume but the Waters 2777C Sample Manager Autosampler software program could load any volume needed by delaying the inlet start method. When 5 mL of 10 ppt EDC contaminated water was injected into the system, then the entire 50 pg enters the SPE and LC separation systems.

2.5.2 AquaAnalysis Method Results

Table 2-2 lists the mass spectrometer optimizations values used for both the automated, on-line method and the manual, off-line method. Appendix A includes more detailed MS settings for both methods.

Table 2-2: MS/MS Optimizations for Estrogenic EDCs

	MW (g/mol)	ESI# Mode	Precursor Ion	Cone Voltage (V)	MRM* Transition (m/z)	Collision (eV)
Estrone (E1)	270.37	(-)	269.3	50	269.3 / 145.3	30
β-Estradiol (E2)	272.38	(-)	271.3	50	271.3 / 145.3	35
Estriol (E3)	288.38	(-)	287.1	55	287.1 / 171.0	35
17β-Ethinylestradiol (EE2)	296.41	(-)	295.2	50	295.2 / 145.2	40
Diethylstilbestrol (DES)	268.35	(-)	267.1	40	267.1 / 251.2	25

#Electrospray Ionization (ESI); *Multiple reaction monitoring (MRM)

Figure 2-3 is the LC chromatogram with the five separate MRM transitions and Table 2-3 presents the retention times, MQLs, MDLs, high concentration curve R^2 and the low concentration curve R^2 for the five MRM transitions. The complete list of method parameters and steps are listed in Appendix A.

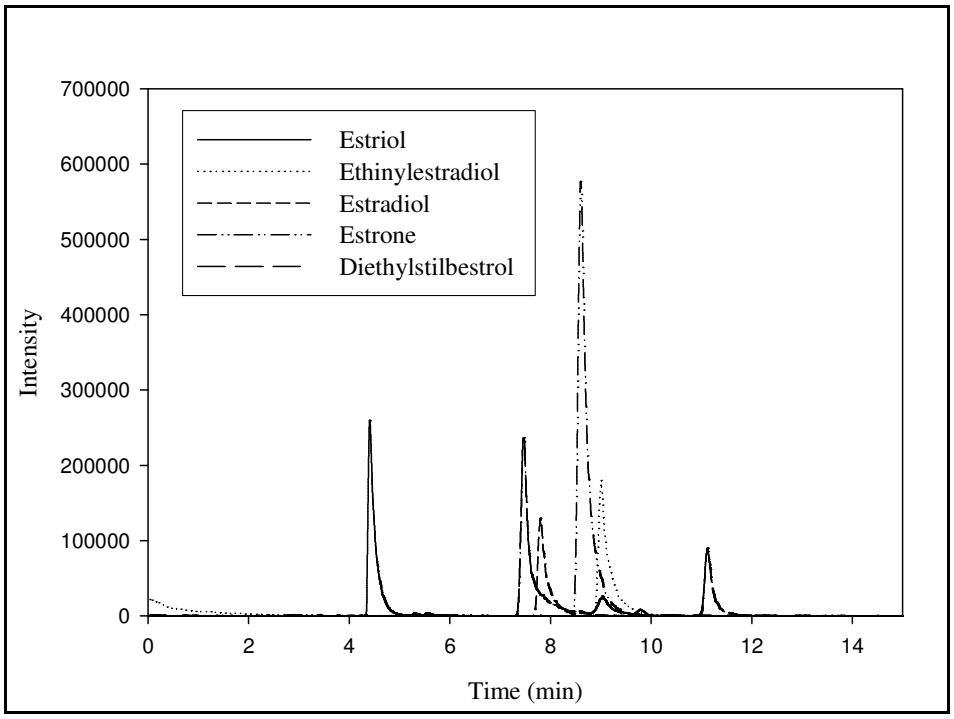


Figure 2-3: Automated, On-line SPE Method Chromatogram with Separated MRMs

Table 2-3: AquaAnalysis Detection Limits for Estrogenic EDCs

	Retention Times (min)	**Minimum Quantification Limits (MQLs)**	**Minimum Detection Limits (MDLs)**	**High Level R^2**	**Low Level R^2**
Estrone (E1)	6.4	5 ppt	2.5 ppt	0.9757	0.7474
Estradiol (E2)	5.9	10 ppt	1.0 ppt	0.9846	0.8318
Estriol (E3)	3.9	2.5 ppt	1.0 ppt	0.9916	0.6908
17β-Ethinylestradiol (EE2)	6.8	10 ppt	2.5 ppt	0.9777	0.9836
Diethylstilbestrol (DES)	6.3 9.2	2.5 ppt	2.5 ppt	0.9920	0.5315

There were two peaks associated with the DES analyte because of the stereoisomeric impurities in the purchased solid stock samples. Both peaks associated with DES were used to determine the concentration, MDL and R^2 by adding the peak areas together, then the analysis was

preformed on the sum. The same procedure was used for the EPA 1694 Method so that results were comparable.

Another value used to understand the difference in methods is the repeatability in sample quantification. The RSD% was calcuated for three injections from the same sample vial at 25 ppt, 50 ppt and 100 ppt. Table 2-4 contains the RSD% for each estrogenic EDC.

Table 2-4: AquaAnalysis Relative Standard Deviations for Repeated Injections

	Relative Standard Deviations between Multiple Injections (%)				
	Estriol	**Estradiol**	**Estrone**	**Ethinylestradiol**	**Diethylstilbestrol**
25 ppt	4.71	4.36	5.59	3.41	5.65
50 ppt	17.59	18.14	28.60	28.07	6.84
100 ppt	5.14	6.14	6.70	4.82	8.52

2.6 EPA 1694 Method

2.6.1 EPA 1694 Method Background

Since the published EPA 1694 Method does not include EDCs, the procedures for the acidic extraction had to be modified for the estrogenic compounds. Deviations from the published method include, not acidifying the raw sample or the preparation of the HLB column and not adding EDTA to the raw sample. These steps were excluded because the methods used in this study consisted of deionized water with known spiked contaminants in order to compare methods.

An initial spiked raw water sample size of 1000 mL was concentrated to 4 mL using the HLB column. It was not apparent as to why the samples in the EPA 1694 Method were concentrated

to 4 mL instead of 1 mL, when the autosampler vials hold 1 mL and a decreased extracted volume leads to increased sample concentration. The HLB extraction cartridges were one-time use only and the complete extraction time was 8 hours in duration. The instrument run time was 16 min using the LC-MS-MS system and an injection volume of 15 μL. Therefore, the extraction of a 10 ppt EDC sample of 1L leads to 2,500 ppt in 1mL sample vials and 37.5 pg injected into the instrument. The concentrations listed for the EPA Method refer to the original sample concentration in 1 L water prior to extraction.

2.6.2 EPA 1694 Method Results

Figure 2-4 is the EPA 1694 Method LC chromatogram with the five separate MRMs and Table 2-5 shows the retention times, MQLs, MDLs, high concentration curve R^2 and the low concentration curve R^2 for the five MRM transitions. The complete list of method parameters and steps are listed in Appendix A.

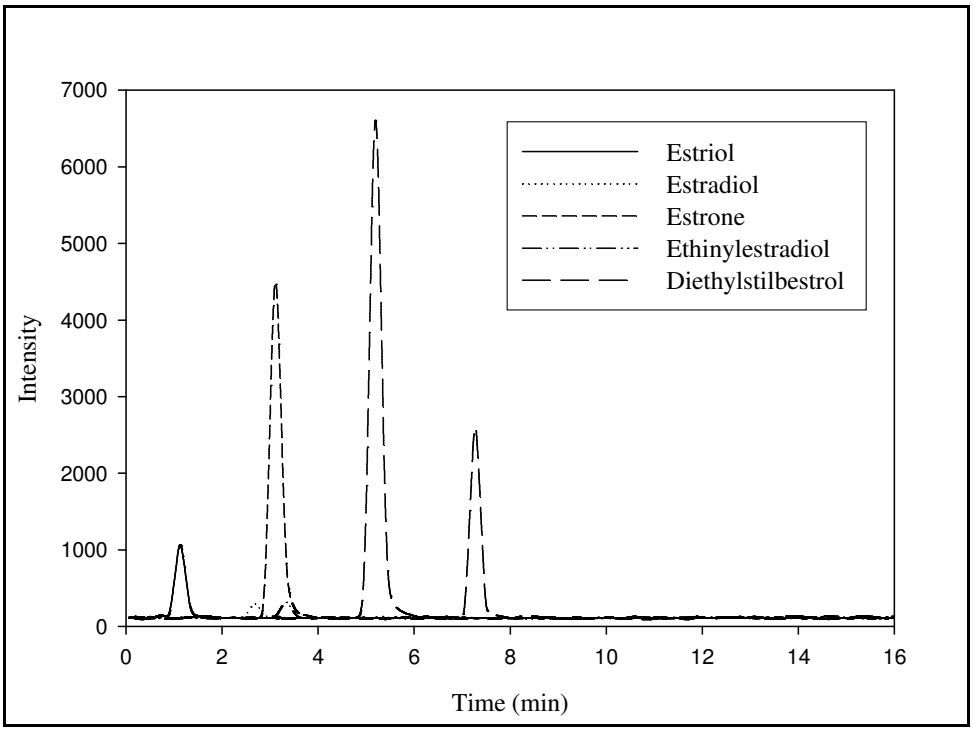


Figure 2-4: Manual Off-line SPE Method Chromatogram with Separated MRMs

Table 2-5: EPA 1694 Method Detection Limits for Estrogenic EDCs

	Retention Times (min)	**Minimum Quantification Limits (MQLs)**	**Minimum Detection Limits (MDLs)**	**High Level R^2**	**Low Level R^2**
Estrone (E1)	3.1	2.5 ppt	1.0 ppt	0.9940	0.8472
Estradiol (E2)	2.7	30 ppt	5 ppt	0.9954	0.7784
Estriol (E3)	1.1	2.5 ppt	1.0 ppt	0.9804	0.8735
17β-Ethinylestradiol (EE2)	3.3	20 ppt	5 ppt	0.9954	0.7784
Diethylstilbestrol (DES)	5.2 7.2	1.0 ppt	1.0 ppt	0.9602	0.8370

The RSD% was calcuated for three injections from the same sample vial at 25 ppt, 50 ppt and 100 ppt. Table 2-6 presents the RSD% for each estrogenic EDC.

Table 2-6: EPA 1694 Method Standard Deviations for Repeated Injections of Estrogenic EDCs

	Relative Standard Deviations between Multiple Injections				
	Estrone	**Estradiol**	**Estriol**	**Ethinylestradiol**	**Diethylstilbestrol**
25 ppt	9.75	173.21*	12.71	173.21*	18.90
50 ppt	16.65	4.00	9.28	14.43	4.03
100 ppt	5.68	16.48	6.38	15.92	7.80

*These RSD% are very large due to both no-dection and small-detection within the same sample.

2.7 Identification of Treatment Byproducts with Optimized Method

2.7.1 Optimized Method Background

The new method was optimized for EE2 and DES at both high and low concentrations. Because of the optimized detection and quantification along with the target levels needed for the byproduct identification experiments, SPE was not needed. Therefore, this method was developed on the Waters Alliance 2695 Separation Module coupled to a Waters Quattro *micro*™ API Quadrupole Mass Spectrometer using a Kromasil® C-18, 3.5µm 4.6x150mm Column. This method also utilized the Waters 2996 Photodiode Array Detector attached to the Alliance prior to MS sample introduction. If SPE was needed, then the AquaAnalysis system would be used. If the manual method was the only one available, then an extraction of 10 ppt EDC in raw water (1 L) should become 10,000 ppt in 1mL sample vials and 1 ng in a 100 µL injection, assuming 100 % recovery.

The previous methods did not provide absorbance chromatograms, which required the identification process to rely solely on the chromatography and MRMs. Since DES and EE2 have distinct absorbance curves with local maxima at different wavelength, the addition of a

PDA decreased the variability in sample signal, by comparing chromatograms, mass spectra and absorbance spectra.

2.7.2 Optimized Method Results

Figure 2-5 and Figure 2-6 are the LC chromatograms of the separate MRMs for EE2 and DES. Table 2-7 shows the retention times, MQLs, MDLs, high concentration curve R^2 and the low concentration curve R^2 for the two MRM transitions. The complete list of method parameters and steps are listed in Appendix A.

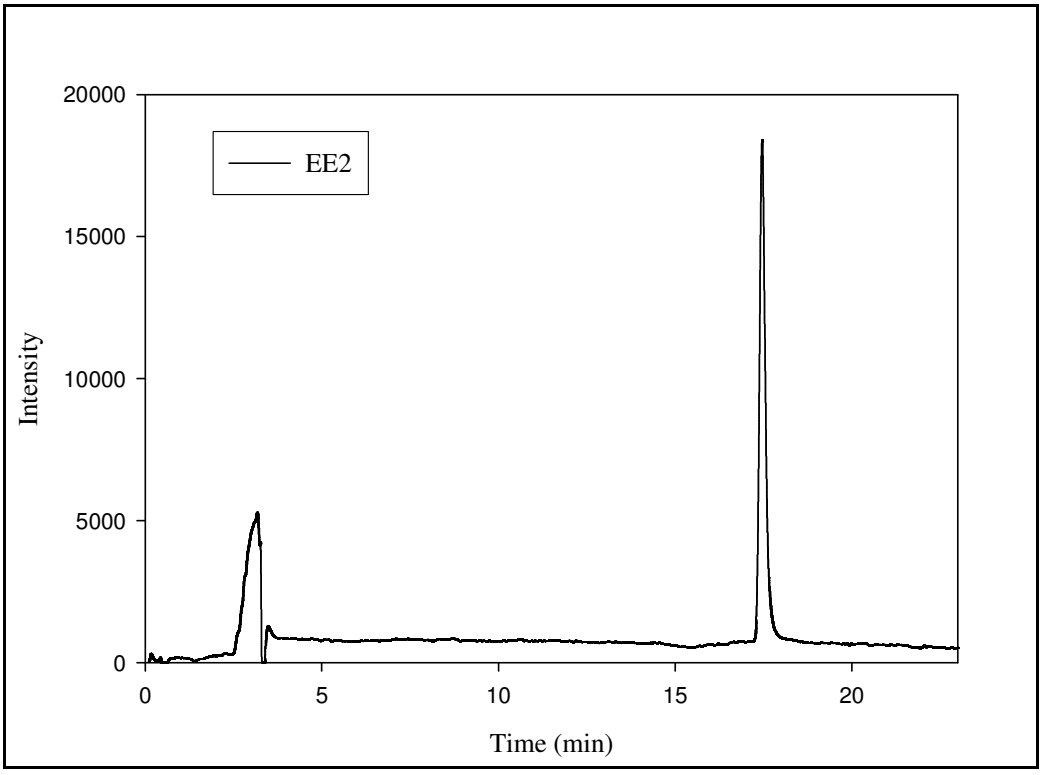


Figure 2-5: Optimized Method Chromatogram with Separated EE2 MRM

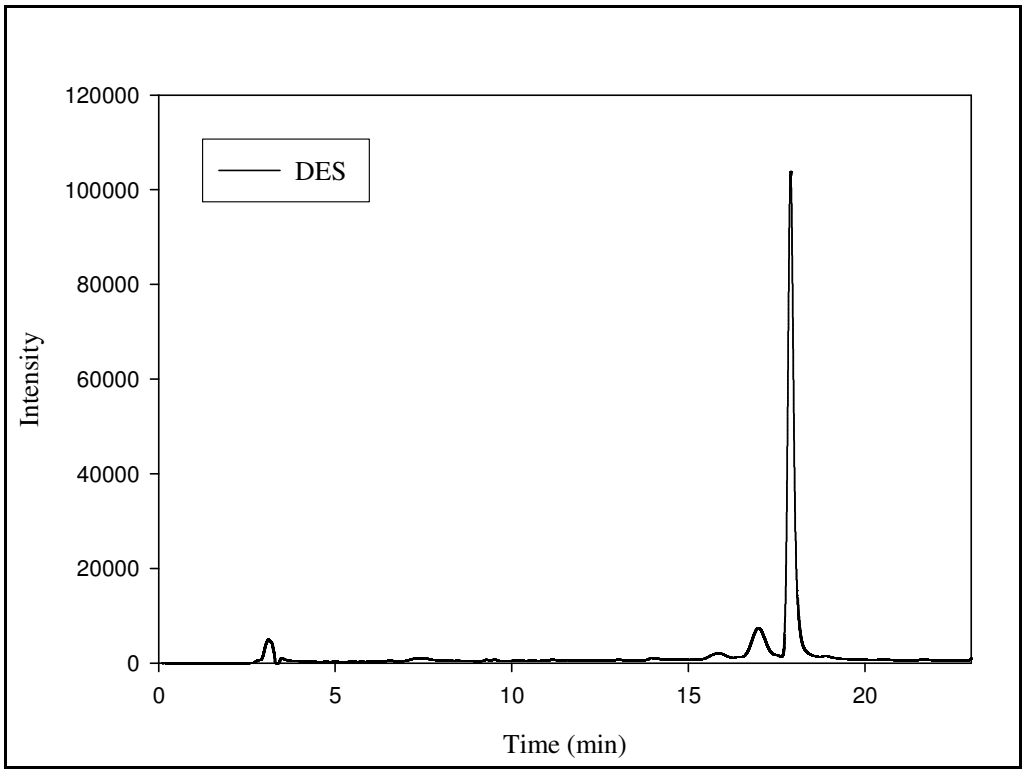


Figure 2-6: Optimized Method Chromatogram with Separated DES MRM

Table 2-7: Optimized Method Detection Limits for Estrogenic EDCs

	Retention Times (min)	**Minimum Quantification Limits (MQLs)**	**Minimum Detection Limits (MDLs)**	**High Level R^2**	**Low Level R^2**
17β-Ethinylestradiol (EE2)	17.49	13.3 ppt (0.045 µM)	13.3 ppt (0.045 µM)	0.9727	0.9880
Diethylstilbestrol (DES)	16.97 17.89	2.68 ppt (0.01 µM)	>2.68 ppt (>0.01 µM)	0.9821	0.9714

The RSD% was calcuated for three injections from the same sample vial at 5 µM, 8 µM and 10 µM. Table 2-8 holds the RSD% for EE2 and DES.

Table 2-8: Optimized Method Standard Deviations for Repeated Injections of Estrogenic EDCs

	Relative Standard Deviations between Multiple Injections	
	17β-Ethinylestradiol	**Diethylstilbestrol**
5 µM	0.72	4.60
8 µM	0.99	4.62
10 µM	1.02	1.66

This optimized method decreased the RSD% significantly compared to the EPA 1694 and AquaAnalysis methods because of the PDA addition. The PDA also decreased the MQLs and MDLs because specific wavelengths could be isolated from the LC chromatogram and then manipulated. The increased sample injection volume was also responsible for the decreased MQLs and MDLs, which is based on the principle that increased sample injection volume will always give an increased signal.

2.8 Discussion of Methods

The manual method was a very labor intensive extraction technique producing MQLs of 30 ppt or lower for the estrogenic EDCs. The AquaAnalysis was less time consuming to operate and produced MQLs of 10 ppt or lower with only 5 mL sample injection. Developing a new method for the AquaAnalysis was time intensive; however, once a method was optimized, then the sample run times decreased dramatically and the instrument could be run when the operator was not present due to the system's automated capabilities. The manual method may have had significantly higher MQLs due to the more intense manipulation of the samples by the operator and due to the systematic error of the multiple step extraction.

The method comparison also showed a decrease in error when using the automated version in the higher concentration range when compared to the manual version because of the elimination of sample transfers and possible contaminants entering the samples throughout the extraction steps. When comparing the low range concentrations, the EPA 1694 Method produced similar detection limits with better reproducibility than the AquaAnalysis system. Both methods reported high R^2 values for the high concentrations, but overall the EPA 1694 Method produced calibration curves at the low concentrations that better fit the linear estimation between the concentrations and the peak areas.

A significant decrease in cost was also noted when using the automated extraction due to the decrease in labor expenditures and in the cost of SPE cartridges. Since the automated method required less raw sample, there was a large reduction in cost for sample collection and shipment even prior to extraction. There was however considerable cost associated with the solvent needed to operate the system compared to the manual method's requirements. The AquaAnalysis system was initially more expensive than the instrumentation for the EPA 1694 Method; however, there was a large savings in decreased labor costs.

Overall, both methods were comparable in analyte detection and quantification with some differences in the performance on individual EDC basis. With the increased efficiency of sample preparation and decreased raw sample volumes, the AquaAnalysis system has high potential in any analytical discipline. There are improvements, however, that were applied to the methods and the instrumentation for both the AquaAnalysis system and the EPA 1694 Method.

The optimized method was produced based on the AquaAnalysis and EPA 1694 Method results and focus shifted towards two analytes at low concentrations and high concentrations, which were required for EDC treatment and byproduct studies. The optimized method decreased sample variability; showed a better fit to a linear calibration with both high and low concentrations; and lower MQLs and MDLs.

2.9 Conclusions

The AquaAnalysis and EPA 1694 methods were comparable in analyte detection and quantification with differences in the performance on individual EDC basis. The AquaAnalysis system promises increased efficiency of sample preparation and decreased raw sample volumes. There were improvements applied to the methods and the instrumentation for both the AquaAnalysis system and the EPA 1694 Method to produce an optimized method.

With the molar absorption coefficients and the absorbance chromatograms, the optimized method decreased sample variability between injections. The optimized method also provided a higher R^2 in the linear calibration with both high and low concentrations. Compared to the AquaAnalysis and EPA 1694 methods, the optimized method also lowered the method quantification limits and the method detection limits.

CHAPTER 3 Endocrine Disrupting Compounds and their Disinfection Byproducts

3 EDCs in Drinking Water Treatment

3.1 Background on EDCs in Drinking Water

The first detection of steroidal hormones in natural waters was documented by Tabak and Bunch in 1970 with a study entitled “Steroid Hormones as Water Pollutants”. These estrogenic contaminants were found to enter the environment by multiple pathways, including the largest source, human and animal excretion. These compounds exit the body in conjugated forms, which are then deconjugated within the wastewater treatment process. After discharge into surface waters, these hormonally active contaminants have the potential of entering drinking water sources.

Even though there is a contentious debate over the impacts of these compounds, it is useful to determine their presence in drinking water, because of the variations in initial concentrations and removal efficiencies. With this in mind, it is incumbent upon the drinking water research community to continue to identify and quantify EDCs to determine possible present and future impacts.

There have been numerous studies devoted to endocrine disruptors and their kinetic reactions during disinfection treatments. Few drinking water treatment techniques for the removal of estrogenic compounds, however, have been compared for treatment effectiveness and byproduct formation. Since the primary goal of disinfection is to remove or inactivate pathogenic microorganisms, the removal of EDCs from drinking water has customarily been on secondary

concern. However, the future consequences on ignoring these contaminants could be significant, even though major effects may not be observed until later generations. It is imperative, therefore, that the parent compounds and the resultant treatment byproducts be identified to determine the specific contaminants, which contribute to the overall estrogenic activity.

Identification of EDCs present in drinking water is an integral step in comparing the removal ability of disinfection techniques. Using liquid chromatography/mass spectroscopy, the identification and quantification of parent estrogenic compounds can be obtained as well as the identification of treatment byproducts and their concentrations relative to the decrease in each parent compound.

The steroidal activity of environmental estrogens can only be accurately determined through bioassays, which target estrogenic hormones. In the absence of bioassays, estrogenic activity can be approximately determined by comparing structures similar to the parent estrogenic compound. By structure alone this class of estrogenic contaminants is present in numerous molecular shapes and sizes; therefore, comparison to the parent compound with its specific moieties is a rudimentary but effective method.

The following sections discuss the possible removal processes that may be employed in the removal of estrogenic contaminants. These selected removal techniques were applied to synthetic estrogens ethinylestradiol and diethylstilbestrol. The synthetic estrogens were selected based on their relative estrogenic strength in comparison to natural estrogens and their observed emergence as contaminants in drinking water (See *Literature Review, Chapter 1*).

3.2 Selected Removal Processes

Numerous treatment techniques are used for drinking water disinfection. The primary goal for disinfection is to protect the public from waterborne diseases by employing water treatments to reduce the concentration of pathogenic organisms through inactivation and/or removal (Crittenden *et al*., 2005). These disinfection techniques are customarily organized into two categories: primary and secondary. Primary disinfection refers to the inactivation of microorganisms and secondary disinfection describes the residual disinfectant applied to water destined for the distribution network. A typical primary disinfectant is a strong oxidizer, while the secondary disinfectant is a weak oxidizer.

The chemical oxidation process is also used to reduce inorganic contaminants, hazardous synthetic organic compounds and active steroidal chemicals (Singer and Reckhow, 2011). The oxidation reactions are heavily dependent on the pH of the system, due to the different oxidation states of the species present at various pHs. The rates of the oxidation reactions are also dependent on the pH, as well as the system temperature, number of species present and types of species present in the reaction. The strength and rate of an oxidation reaction can also determine which disinfectant is used in a treatment system, based on system limitations and requirements, such as required disinfectant concentration and contact time (C·t). Taste and odor problems can also be addressed by the use of chemical oxidation.

A widespread concern in using chemical oxidants is the formation of disinfection byproducts (DBPs). The choice of oxidant implemented in water treatment plants is directly related to the

raw water quality, including total organic carbon, organic halides, and type of microbiological activity. The EPA has set limits on selected chlorination DBPs due to the carcinogenic qualities of many of these contaminants (*Safe Drinking Water Act: EPA 816-F-09-0004*, 2009).

This research focused on chlorination, chloramination and ozonation disinfection treatment processes. There are numerous other techniques that could be used in the removal of EDCs through drinking water treatment; however, the selected chemical disinfection processes are the most widely used disinfection treatment techniques.

Figure 3-1 shows a simplified schematic of drinking water treatment. The disinfection step was the process studied in this research.

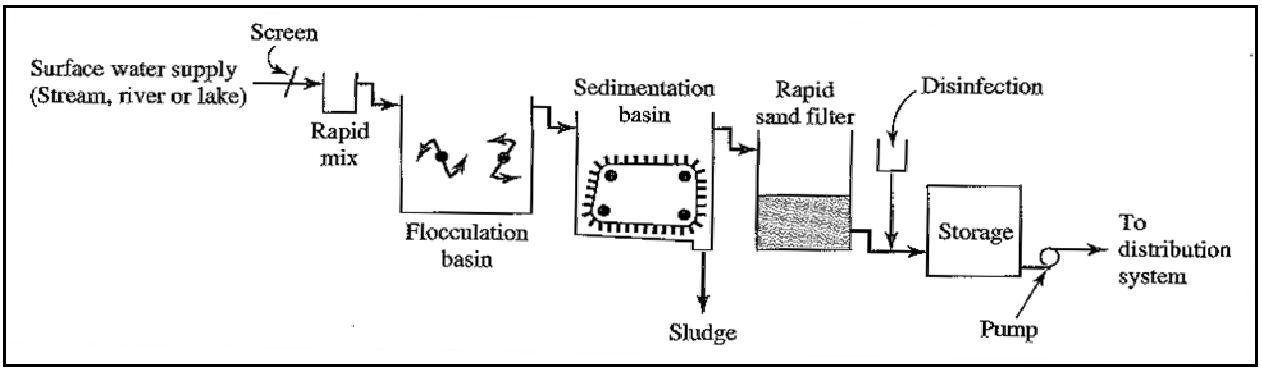


Figure 3-1: Diagram of Drinking Water Treatment Plant (Davis and Masten, 2004)

3.2.1 Chlorination

Chlorination is the most widely used disinfection process in drinking water treatment in the United States. Chlorine is a strong oxidizing agent that is operator-friendly, inexpensive and a

reliable disinfectant. For drinking water, chlorine can be introduced as either chlorine gas ($Cl_{2(g)}$), sodium hypochlorite (NaOCl) or calcium hypochlorite ($Ca(OCl)_2$) (Masters, 1997).

When chlorine gas is added to pure water, the species resulting from the dissociation of Cl_2 are hypochlorous acid (HOCl) and hypochlorite ion (OCl^-). The primary disinfecting agent in the chlorination process is HOCl, while OCl^- is a less effective disinfectant. The pKa for HOCl is 7.54; therefore, the strength of the disinfecting agent present is based on the pH and the corresponding chlorine oxidation state. The reaction temperature also dictates the rate of the reaction, with warmer water causing the dissociation of hypochlorous acid to occur at a slightly lower pH (Crittenden *et al.*, 2005). The dissociation equilibria for chlorine in water are shown below.

$$Cl_2 + H_2O \rightleftharpoons HOCl + Cl^- + H^+ \rightleftharpoons ClO^- + H^+ \qquad \textbf{Reaction 1}$$

The primary advantage of using chlorine as a disinfectant is that after the initial reaction, the treated water can retain a chlorine residual, which can help protect the quality of drinking water throughout the distribution system. According to the Safe Drinking Water Act, regulated by the EPA, the maximum allowable level of chlorine residual as Cl_2 is 4.0 mg/L. With high levels of Cl_2 residual, there is potential for irritation to eyes and nose, as well as stomach discomfort following long term exposure (SDWA, 2009).

Even though chlorine is relatively stable in pure water, it has a slow reaction rate with natural organic matter (NOM) found within drinking water. There have been numerous studies on

chlorination DBPs; however, there are no fundamental (i.e. non-empirical) reaction rates available for HOCl and NOM because of the variability in NOM composition (Crittenden, 2005).

The disadvantage of using chlorine as a disinfectant is the formation of trihalomethanes (THMs) and haloacetic acids (HAAs), as well as other chlorination DBPs. These chlorinated DBPs have been implicated in damaging internal organs and increasing the risk of cancer (Masters, 1997). Since these DBPs are formed from a reaction between natural organic matter and chlorine, the concentration of THMs and HAAs is directly proportional to the amount and type of natural organic matter in the treated water. Therefore, THM and HAA concentrations can be decreased with organic removal during treatment before chlorination (Morris and Isaac, 1981).

The EPA drinking water regulations (EPA 816-F-09-0004) for the presence of DBPs has been set at MCLs of 0.060 mg/L for HAA5 and 0.080 mg/L for total THMs (SDWA, 2009). The TTHMs, and the corresponding MCLs, include bromodichloromethane (zero), bromoform (zero), dibromochloromethane (0.06 mg/L), and chloroform (0.07 mg/L), where the MCLGs are given in parentheses (SDWA, 2009). The five haloacetic acids in HAA5, and the corresponding MCLs, include dichloroacetic acid (zero), trichloroacetic acid (0.02 mg/L), monochloroacetic acid (0.07 mg/L), bromoacetic acid (N/A) and dibromoacetic acid (N/A). With long-term exposure, HAA5s increase risk of cancer and TTHMs are implicated in liver and kidney failure, nervous system problems and increased risk of cancer (EPA, 2009).

Since chlorination is the most widely used disinfection process, there have been numerous studies on the treatment byproducts resulting from reactions of free chlorine with EDCs. The

chlorination of EE2 has produced numerous byproducts that retain the estrogenic qualities of the parent compound, with the major byproducts being identified as 4-chloro EE2 and 2,4-dichloro EE2 (Alum *et al.*, 2004; Hu *et al.*, 2003; Moriyama *et al.*, 2004).

The Deborde *et al.* (2004) study determined the reaction rates for the chlorination of EE2, where $k_1 = 2.04 \times 10^5\ M^{-2}s^{-1}$; $k_2 = 4.33\ M^{-1}s^{-1}$; $k_3 = 3.52 \times 10^5\ M^{-1}s^{-1}$, for the following second order reactions:

$$\begin{aligned} ED + HOCl + H^+ &\rightarrow \text{byproducts} \quad k_1 \\ ED + HOCl &\rightarrow \text{byproducts} \quad k_2 \\ ED^- + HOCl &\rightarrow \text{byproducts} \quad k_3 \end{aligned}$$

Reaction 2

Deborde *et al.* found $t_{1/2}$ of 73.2 min for the removal of EE2 with a chlorine dose of 0.1 mg/L and $t_{1/2}$ of 7.3 min for the removal of EE2 with a chlorine dose of 1 mg/L (2004).

There has been no published research on the chlorination of DES that discusses the reaction rates or the byproducts produced, along with the respective estrogenic qualities of the byproducts; however, Lee and Morris (1962) published kinetics of the chlorination of phenol. This publication was used along with Larson and Weber's (1994) discussion on chlorination of phenols to determine the identities of the byproducts produced for both the chlorination of EE2 and DES. Since a lack of data is available, it is necessary to identify the byproducts and their relative concentrations prior to determining their estrogenic qualities and/or their formation rates.

3.2.2 Chloramination

Chloramination reactions with estrogenic contaminants have not been studied as thoroughly as other oxidation reactions used in drinking water treatment. To increase the lifetime of the residual chlorine disinfectant, some drinking water utilities add ammonia to finished water, forming chloramines; monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_3). There are four types of chloramination, which differ based on the order of ammonia and free chlorine introduction: pre-ammoniation, pre-chlorination, simultaneous addition and pre-formed chloramines.

Chloramines have a much lower reaction rate compared to the chlorination reaction rates with organic contaminants and are typically used as a secondary disinfectant. The primary purpose for chloramine addition is to control microbes (EPA, 2009). There are potential long-term chloramine exposure effects, such as anemia, eye and nose irritantation and stomach discomfort (EPA, 2009). According to the SDWA, the MRDL for chloramines as Cl_2 is 4.0 mg/L.

$$
\begin{aligned}
2H_2O + Cl_{2(g)} &\rightleftharpoons 2HOCl + 2H^+ \\
HOCl + NH^+_4 &\rightleftharpoons NH_2Cl + H_3O^+ \\
NH_2Cl + HOCl &\rightleftharpoons NHCl_2 + H_2O \\
NHCl_2 + HOCl &\rightleftharpoons NCl_3 + H_2O
\end{aligned}
$$

Reaction 3

Since the published research on chloramination reactions with estrogenic compounds is limited, daughter products formed from the reactions have not been identified for any estrogenic EDCs. If the chloramine formation rate is slower than the free chlorine reaction rate with the estrogenic compounds, then the same daughter products will be produced as discussed in the free chlorine

reaction. Conversely, if the chloramine formation rate is faster than the free chlorine reaction rate with the contaminant, then new daughter products may be formed. Since the reaction rates and products for chlorine and ammonia are pH dependent, the daughter products produced will consequently depend on the pH of the system.

The reaction rate constant between the un-ionized species HOCl and NH_3 is 4.17 x 10^6 $M^{-1}s^{-1}$ at pH 7 and 25 ^oC (Morris and Isaac, 1981) compared to the apparent reaction rate constant of HOCl and ethinylestradiol at pH 7 and 20 ^oC of 1.12 x 10^2 $M^{-1}s^{-1}$ (Deborde *et al.*, 2004). Even though the comparison of reaction rates indicates that the byproducts will not form, there is potential for byproducts to be observed based on the order of ammonia and free chlorine additions; specifically pre-chlorination and simultaneous addition chloramination reactions.

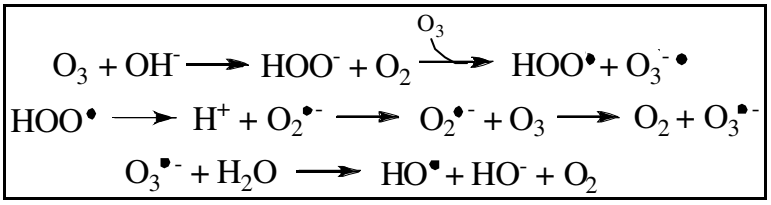
There is no published research on the chloramination of DES or EE2. Chloramination is widely used, however, as a secondary disinfectant and could result in byproduct formation within a distribution system. The reaction rates with chloramines are relatively slow compared to other types of disinfectants.

3.2.3 Ozonation

Ozonation involves the dissolution of ozone (O_3) in water, providing a very powerful disinfectant that is even more effective against cysts and viruses than chlorine (Masters, 1997). Ozone also has the added advantage of leaving only inconsequential taste or odor problems. Although ozonation is widely used in European water treatment facilities, it has the disadvantage of not forming a protective residual in the treated water and the process is more expensive than

chlorination. The level of bromate in drinking water is regulated, thus ozone can not be used if high levels of bromide are present in the water source. Compared to chlorination, the use of ozone decreases trihalomethane formation, and helps control taste and odor problems (Viessman and Hammer, 2005).

The following reactions show the intermediates in the ozone reaction with water (Masters, 1997).



Reaction 4

Reactions with ozone and EDCs are quite rapid as well. Huber *et al.* (2003) presented a second order rate constant for the reaction of ozone with 17α-ethinylestradiol that is near the diffusion limit. Given the pK_a of the phenolic moiety, ethinylestradiol would degrade in a fraction of a second, even in the presence of a low ozone residual due to the destruction of the ring structure by oxygen radicals. With similar structures at the point of attack, it is expected that other estrogens are similarly reactive with ozone; however, Huber *et al.* (2004) found estrone to have a decreased removal efficiency in wastewater as compared to either estradiol or ethinylestradiol.

The potential toxicity of EDC degradation byproducts have been studied only after ozonation where the phenol ring remained intact and the estrogenic activity was decreased (Huber *et al.*, 2004). The Deborde *et al.* (2005) study determined the reaction rates by competition kinetics for

the ozonation of EE2, where $k_1 = 1.83 \times 10^5\ M^{-1}s^{-1}$ and $k_2 = 3.65 \times 10^9\ M^{-1}s^{-1}$, for the following second order reactions:

$$\begin{aligned} ED + O_3 &\rightarrow \text{byproducts} \quad k_1 \\ ED^- + O_3 &\rightarrow \text{byproducts} \quad k_2 \\ ED^{-2} + O_3 &\rightarrow \text{byproducts} \quad k_3 \end{aligned}$$

Reaction 5

Hashimoto *et al.* found 90 % removal of EE2 with an ozone dose of 1mg/L and 100 % degradation of EE2 with a dose of 3 mg/L O_3 (2006); however, byproducts were not identified in this study.

The ozonation of EE2 has been shown to produce numerous byproducts that maintain the estrogenic activity of the parent compound by retaining the phenolic moiety, which acts as the estrogen receptor binding site (Deborde *et al.* 2005; Dodd *et al.*, 2006; Huber *et al.*, 2005; Nakonechny *et al.*, 2008). Lee *et al.*, 2008 suggest that a hydroxyl group is added to the phenolic ring during preliminary ozonation and can be further ozonated to produce quinones on the same ring structure. They also propose a structure with the phenolic ring broken, creating muconic EE2. These theoretical suggestions of byproduct structures are the same as found in the Huber *et al.* (2004) and Zhang *et al.* (2006) studies; however, Zhang *et al.* determined the EE2 ozonation byproducts using a GS/MS and Huber *et al.* used LC/MS/MS for positive identification of the actual byproducts.

There is no published research on the ozonation of DES available. Since DES has a relatively strong estrogenic activity, it is important that the ozonation byproducts be identified.

3.3 Other Removal Processes

There are many additional techniques that have been evaluated for their effectiveness in removing endocrine disruptors from drinking water. The following processes are not explored in this body of work, but it should be noted, they are equally as important as chlorination, chloramination and ozonation.

3.3.1 Ultraviolet Irradiation

Ultraviolet (UV) irradiation is an established technique for inactivating pathogens in wastewater. An advantage of UV over other some water treatment techniques is that no chemicals are added to the water and UV treatment does not lead to any known toxic byproducts (Mitch *et al.*, 2002). UV has been used in treating photo-reactive chemicals and recent studies suggest that UV may be a possible treatment for EDCs (Rosenfeldt *et al.*, 2004). Previous studies of EDCs using conventional chlorination treatment have shown that estrogenic activity in some cases increases (Hu *et al.*, 2002). Whether this occurs with UV treatment is presently unknown. The potential toxicity of E1 degradation products has been studied only after ozonation in which the phenol ring remained intact and the resulting estrogenic activity decreased (Huber *et al.*, 2004).

3.3.2 Chlorine Dioxide Oxidation

Research conducted by Lee *et al.* (2008) focused on the oxidation reaction of steroid estrogens and chlorine dioxide. The second-order reaction rate constant found for ethinylestradiol and ClO_2 was 1.83 x 10^5 $M^{-1}s^{-1}$ at a pH of 7 and a temperature of 20 ^oC. This reaction is slower than that observed with EE2 and ozone; however, the reaction rate observed for chlorination of EE2 is slower compared to the ClO_2 reaction (Deborde *et al.*, 2004; Deborde *et al.*, 2005).

The initial byproducts formed in the reaction of EE2 with ClO_2 were possibly due to the hydroxyl functional group from the phenolic moiety that donates electrons to the aromatic ring. Since the aromatic ring can accommodate additions of atoms with low electron density, the compounds containing a phenolic ring are highly reactive; therefore, the first reaction sites will be located on the aromatic ring.

3.3.3 Sorptive Filtration

Filtration systems have demonstrated varied abilities in EDC removal stemming from the differences in chemical properties of each compound. The most promising type of filtration that has been studied to remove EDCs and PPCPs is riverbank filtration (Storck *et al.*, 2010). Riverbank filtration is a low cost and low maintenance treatment technique that can be used as the first step to decontaminate surface waters. The effectiveness of this treatment technique is based on the sorption capacity of the bank sediments, sorption and desorption behavior of contaminants, biodegradation ability and rate of flow through the sediments (Storck *et al.*, 2010).

3.3.4 Coagulation

Coagulation studies of estrogenic contaminants, including Westerhoff *et al.* (2005), show that low removal levels are achieved. When a disinfectant is added to the coagulation treatment (e.g. chlorine or ozone, etc.), the removal rate levels increased with respect to the removal rate of the oxidant used for disinfection. Since coagulation is a physical treatment of the contaminants, no daughter products will be formed. With coagulation plus a disinfectant, the daughter products formed are only a result of the reaction between the disinfectant and the contaminant.

3.3.5 Biological Treatment

Biological treatment processes are not usually selected for drinking water treatment; however, research has been done on the biological removal of estrogenic compounds found in wastewaters and many daughter products have been identified. Research pertaining to biological treatment applications for drinking water has increased due to the effectiveness and sustainability of the treatment systems, according to research conducted by Brown (2007). Biological treatment of estrogenic compounds in drinking water will most likely increase due to the relatively high removal rates obtained from biological treatment of wastewater.

3.4 Comparison of Treatment Effectiveness

3.4.1 Experimental Design

The treatment efficacies were compared (1) using the percent degradation of the parent EDC, (2) determining the number of byproducts produced from treatment with ring-structure intact, and (3) estimating the quantification of each byproduct produced. Various contact time intervals,

including instantaneous, short and long term contact, were used to compare the treatments. The initial byproducts formed from the reactions were found with near instantaneous contact (on the order of seconds). To simulate the contact time used in drinking water treatment plants, a short contact time was selected for each type of disinfection, excluding chloramination because this treatment is typically used as a secondary disinfectant. Long contact times of 48 hours were used to simulate the distribution system, with the exclusion of ozonation because the treatment is typically only used as a primary disinfectant. Typical C·t values were chosen based on in-practice values and for applicable comparisons of the differences in selecting disinfection types (Kawamura, 2000). Table 3-1 provides a summary of the dose and duration for each treatment, with an initial EDC concentration of 10 ±0.1 μM for.

Table 3-1: Summary of Treatment Types and Doses

Treatment Type	Instantaneous Contact		Short Contact		Long Contact*	
	Dose	Time	Dose	Time	Dose	Time
Chlorination	2.5 mg/L	0-1 hr	1.5 mg/L 3.0 mg/L 6.0 mg/L	5 hrs	1.5 mg/L 3.0 mg/L 6.0 mg/L	48 hrs
Chloramination (4mg-Cl_2/mg-NH_4Cl)	2.5 mg/L Cl_2 0.6 mg/L NH_4Cl	0-1 hr	1.5 mg/L 3.0 mg/L 6.0 mg/L	5 hrs	1.5 mg/L 3.0 mg/L 6.0 mg/L	48 hrs
Ozonation	0.05 mg/L 0.1 mg/L 0.2 mg/L 0.3 mg/L 0.4 mg/L 0.5 mg/L	Rxn time	1.5 mg/L 2.0 mg/L 3.0 mg/L 6.0 mg/L	Rxn time	Not Applicable	

*Long and short contact for chloramination was preformed as pre-chlorination addition because this option is most widely used in treatment systems.

The instantaneous ozone reaction consisted of doses with a molar ratio of less than or equal to one and the short contact time had doses with a larger than one molar ratio. This ozonation

experimental design was implemented due to the rapid ozone reactions and the need to observe instantaneous byproducts.

3.4.2 Analytical Methods

An analytical method was optimized for EE2 and DES at both high and low concentrations. The method was developed on the Waters Alliance 2695 Separation Module coupled to a Waters Quattro *micro*™ API Quadrupole Mass Spectrometer using a Kromasil® C-18, 3.5 µm 4.6 x 150 mm Column. This method also utilized the Waters 2996 Photodiode Array Detector (PDA) attached to the Alliance prior to MS sample introduction. A detailed discussion of methods used is provided in the *Chapter 2 Analytical Methods*.

Byproduct identification utilized (1) the byproduct polarity relative to the parent compound, (2) the molecular weight and fragmentation pattern obtained from the mass spectrum, (3) the type of disinfection agent, (4) the isotope pattern observed on the mass spectrum, and (5) the absorbance of the byproduct at the local absorbance maximum of the parent compound.

3.4.3 Research Objectives

From the wide variety of pollutants considered as potential EDCs, the focal point of this research was on a select contaminant group due to their endocrine-disrupting potential and their presence in natural water, as reported in several previous studies (Fent *et al.*, 2006; Huber *et al.*, 2004; Richardson, 2002). This research focused on 17β-ethinylestradiol and diethylstilbestrol.

Given the increasing problem of estrogenic EDC contamination in source waters, the purpose of this research was to determine which techniques were the most effective in removing EDCs and which introduced the least amount of harmful byproducts into the water supply system. In order to determine which treatment was the most effective, each process was examined as to the percent degradation of the EDCs and the number of byproducts produced. These experiments were conducted on an individual EDC basis due to the differences in side chain moieties associated with each EDC (Fent *et al.*, 2006; Larson and Weber, 1994). After degradation occurred, the byproducts were identified to determine if the ring structure from the parent compound remained intact. Once the byproducts were identified, the treatment processes were compared using (1) the dose applied, (2) the number of byproducts formed and (3) the relative quantification of the treatment byproducts.

3.5 Experimental Results for the Treatment of Synthetic EDCs

Numerous studies have focused on the disinfection byproducts of ethinylestradiol; however, there have been few studies examining the reactions of diethylstilbestrol with disinfectants and the resulting byproducts. The following sections present the results for the reactions of EE2 and DES with each of the disinfectants selected.

3.5.1 Free Chlorination

Procedures for the chlorination of EE2 and DES are provided in UMass Chlorination Standard Operating Procedures with specific experimental methods listed in Appendix B. These batch

reactions were standardized to pH of 7.0 ± 0.1 and temperature 20 ± 1.0 ^{o}C, so that hypochlorous acid (HOCl) was the dominant free chlorine species for chlorination.

Chlorination consisted of three experimental designs; instantaneous, short term and long term. Each experiment was conducted with excess Cl_2 doses, as compared to the EDC initial concentration of 10 ± 0.1 µM. For the instantaneous reactions, the free chlorine level used was 2.5 mg/L. Doses of 1.5 mg/L, 3.0 mg/L and 6.0 mg/L were used for the short and long term experiments. The short term experiments had reaction times of 5 hrs and the long term experiments had a reaction time of 48 hrs. Samples from the 5 and 48 hrs experiments were placed in 20 ^{o}C environment to maintain constant temperature throughout the reaction period.

Kinetic models of the instantaneous reactions were not included in this study due to the emphasis on the byproduct identification. Rate constant values are available from previous studies (Deborde *et al.*, 2004). Free chlorine was not measured during the instantaneous reactions due to the short reaction times and the duration of the titration process being longer than the reaction interval. Free chlorine was measured in the short and long term experiments to determine the amount of chlorine residual remaining following the reactions.

3.5.1.1 Chlorination of EE2

Reactions

For each chlorination reaction, it was observed that as EE2 was degraded, the total peak area of the byproducts increased. The peak area was determined by manual integration of peaks isolated at 280 nm on the absorbance chromatogram. As the free chlorine dose was increased, the EE2

concentration decreased in proportion to the amount of free chlorine added for the disinfection reaction.

Figure 3-2 displays EE2 and total byproducts peak areas versus time, which resulted from the instantaneous chlorination of EE2. Each experiment included initial concentrations of EE2 of 10.0 ± 1.0 µM.

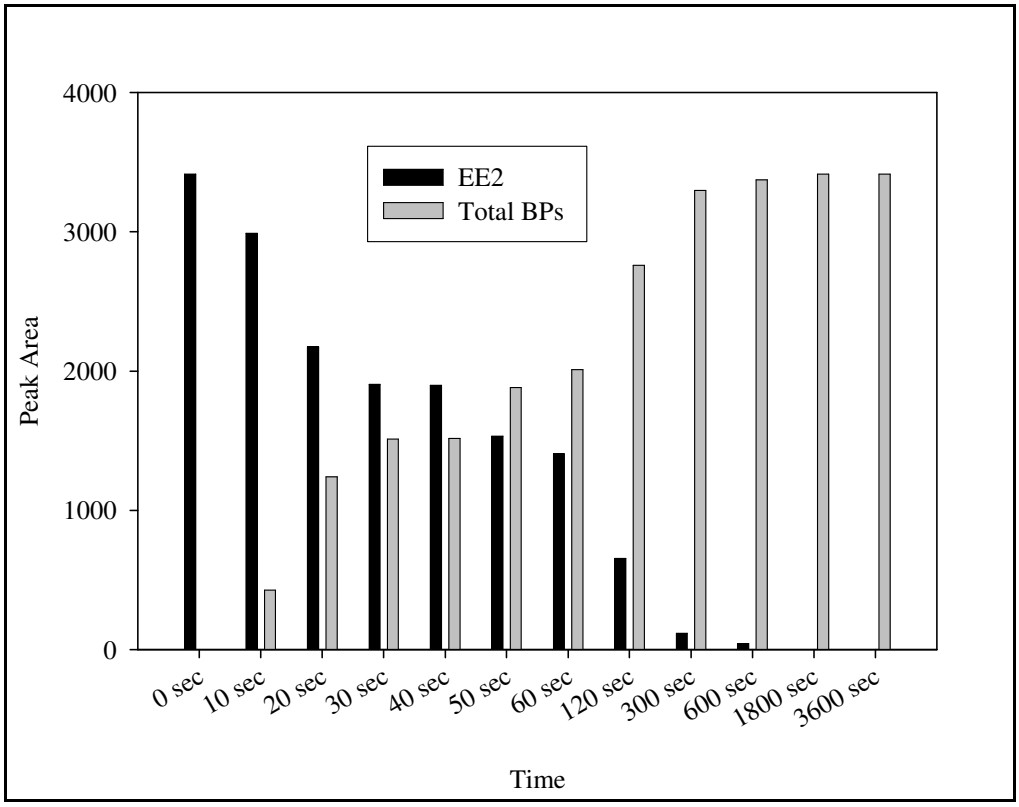


Figure 3-2: Instantaneous Chlorination of Ethinylestradiol

Results for each byproduct separated for individual time increments are presented in Appendix B. The byproducts formed during the instantaneous reactions of EE2 were identical to those

observed in the short term and long term reactions. The chlorine residuals found for the EE2 long and short term chlorinations are also presented in Appendix B.

Detection

EE2 followed an exponential decay function, where the contaminant decreased significantly in early time intervals and with small changes in concentration after 2 min of reaction time. At time 1800 sec, EE2 was not detected by LC/MS; therefore, it was assumed that nearly 100 % of the concentration was degraded. The major byproducts formed from EE2 and Cl_2 reaction were observed at 13, 14 and 19 min of elution time on the chromatogram based on absorbance at 280 nm. Two minor byproducts were found at 15 and 16 min on the chromatogram; however, there was insufficient information available to determine the precise indentity of the minor byproducts of EE2 and free chlorine.

According to the the absorbance spectrum and the molar absorption coefficients for EE2, the maximum absorbance wavelength is at 280 nm. The absorbance shows a characteristic phenolic, multiple-ring pattern of two local maxima between 190 nm and 400 nm. It was therefore assumed that any byproducts detected having an absorbance at 280 nm and showing a similar absorbance pattern to EE2, would still have the parent ring structure intact. Therefore, the PDA was used to isolate specific wavelengths on the total ion chromatogaphs. Figure 3-3 shows the liquid chromatogram for isolated absorbance at 280 nm for the long term chlorination of EE2. Instantaneous and short term chlorination chromatograms are found in Appendix B.

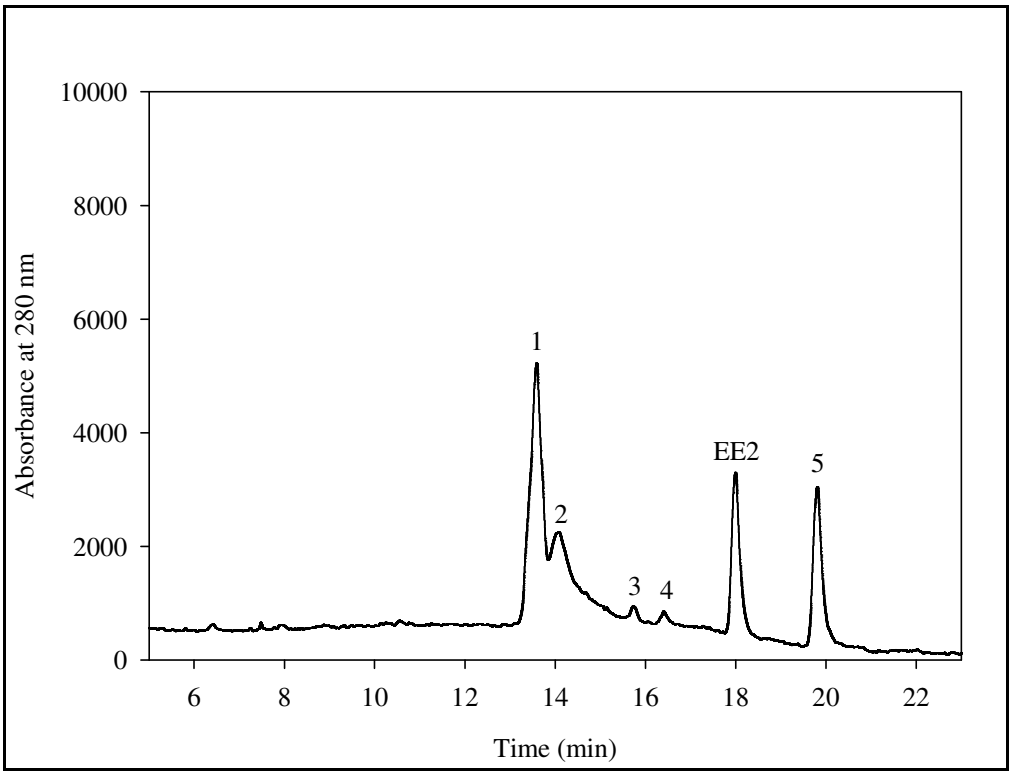


Figure 3-3: Long Term Chlorination of Ethinylestradiol LC Chromatogram with Isolated 280 nm Absorbance

Each peak on the UV_{280nm} chromatogram was integrated to determine the associated areas. The chlorination reactions produced five new byproducts (BPs), with the major BPs eluted at 13, 14 and 19 min. The change in peak areas versus time for the three major BPs and EE2 are shown in Figure 3-4 for the instantaneous chlorination reaction.

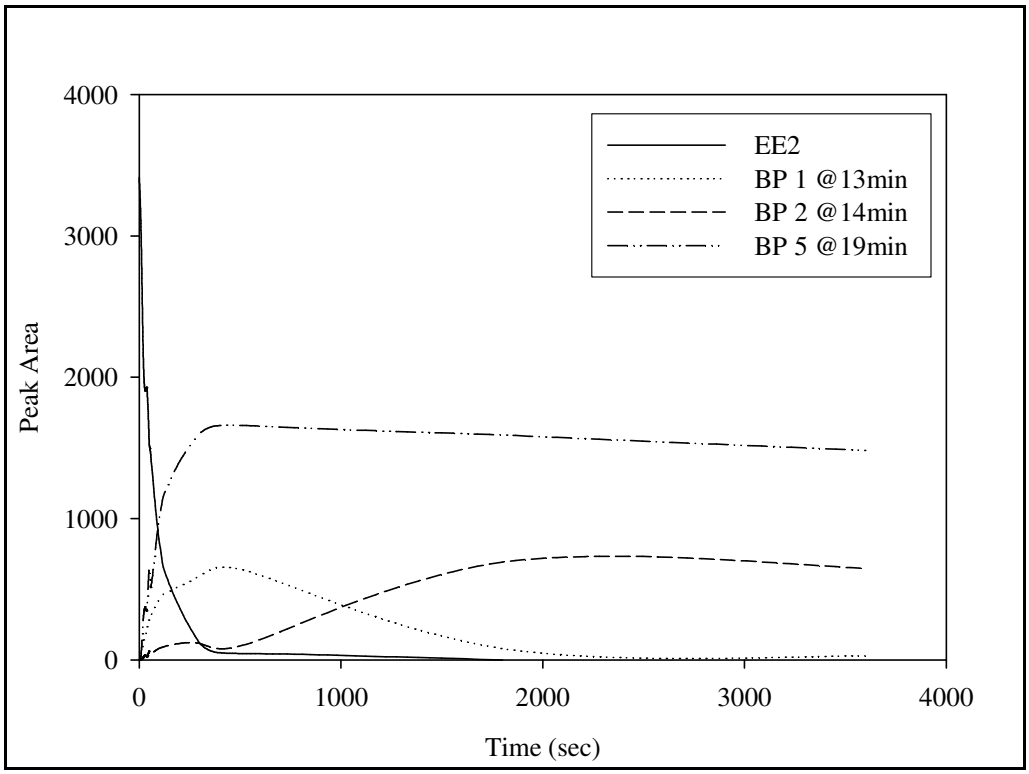


Figure 3-4: Formation of Major Byproducts from Chlorination of Ethinylestradiol

As the reaction time progresses, EE2 follows an exponential decay function. The byproduct 1 at 13 min was formed immediately after the chlorine dose was administered, then started to decrease after 600 sec of reaction. The byproduct 2 at 14 min formed at a slower rate and yielded a lower concentration as compared to the byproduct 1 at 13 min; however, byproduct 2 (14 min) was present within each instantaneous reaction time interval. The byproduct 5 at 19 min was formed immediately after the chlorine dose was introduced to the solution and was present in each instantaneous sample.

Identification

The chlorination of EE2 was performed, finding three dominant byproducts and two minor byproducts with ring structures still intact. The diode array spectrophotometer was used to determine if the byproducts absorbed at 280 nm, which is the relative maximum absorbance of EE2. Figure 3-5 shows the carbon numbering format for EE2 to help in identification of carbon atoms within each moiety.

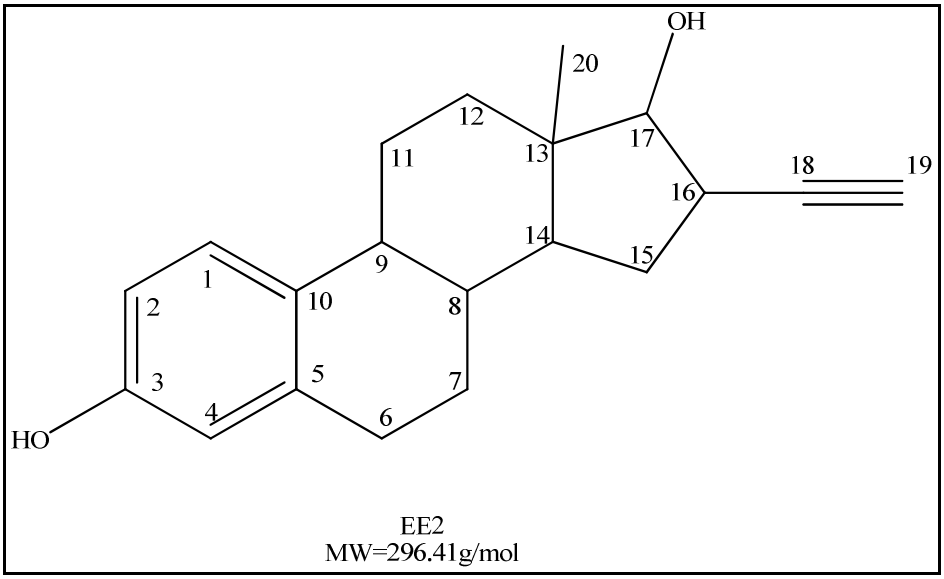


Figure 3-5: Ethinylestradiol

The absorbance spectra for EE2 and the observed chlorination byproducts are shown in Figure 3-6. The byproducts 1 through, 4 found at 13, 14, 15 and 16 min, show the same two local absorbance maxima pattern as EE2 and elute prior to EE2 when using a mobile phase of methanol and water. The byproduct 5 at 19 min contains only one local maximum in the corresponding absorbance spectrum and elutes after EE2.

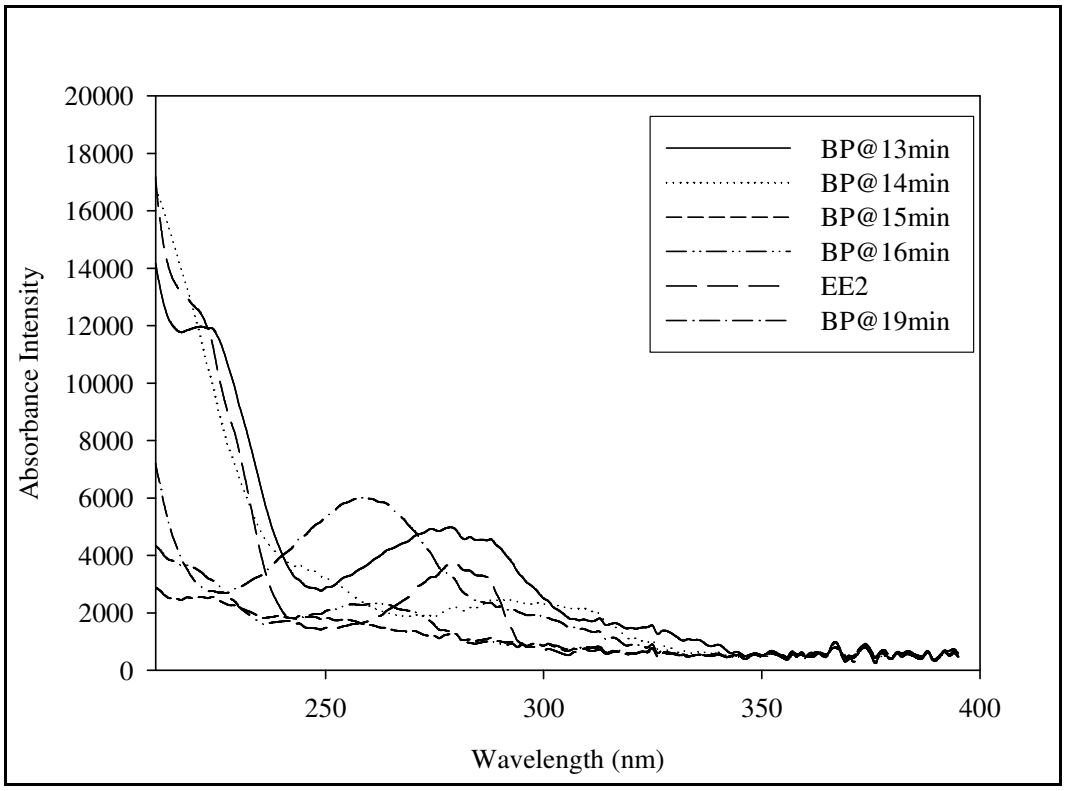


Figure 3-6: Absorbance of EE2 and Chlorination Byproducts

Based on the retention times and corresponding polarity, mass spectra and absorbance at 280 nm, the major byproducts were identified for the chlorination of EE2 and presented in Table 3-2. The mass spectra for each byproduct are located in Appendix B.

Table 3-2: Proposed Identification of EE2 and Chlorination Byproducts

Analyte	Retention Time (min)	Identity
EE2	17 min	EE2
Byproduct 1	13 min	2-chloro EE2 or 4-chloro EE2 (MW 331 m/z)
Byproduct 2	14 min	2,4-dichloro EE2 (MW 365 m/z)
Byproduct 3	15 min	$C_{20}H_{23}O_3Cl$ MW 346 m/z
Byproduct 4	16 min	$C_{20}H_{21}O_2Cl_3$ MW 398 m/z
Byproduct 5	19 min	$C_{20}H_{22}O_3Cl_2$ MW 380 m/z

The proposed reactions between free chlorine and EE2 are shown in Figure 3-7. The major byproducts, 4-chloro EE2, 2,4-chloro EE2 and MW 380, were immediately formed at the introduction of HOCl into a solution of EE2, attacking the phenolic moiety at the ortho position(s), with respect to the hydroxyl group at C3. The minor byproducts could not be accurately identified on the mass spectra due to the lack of molecular ions.

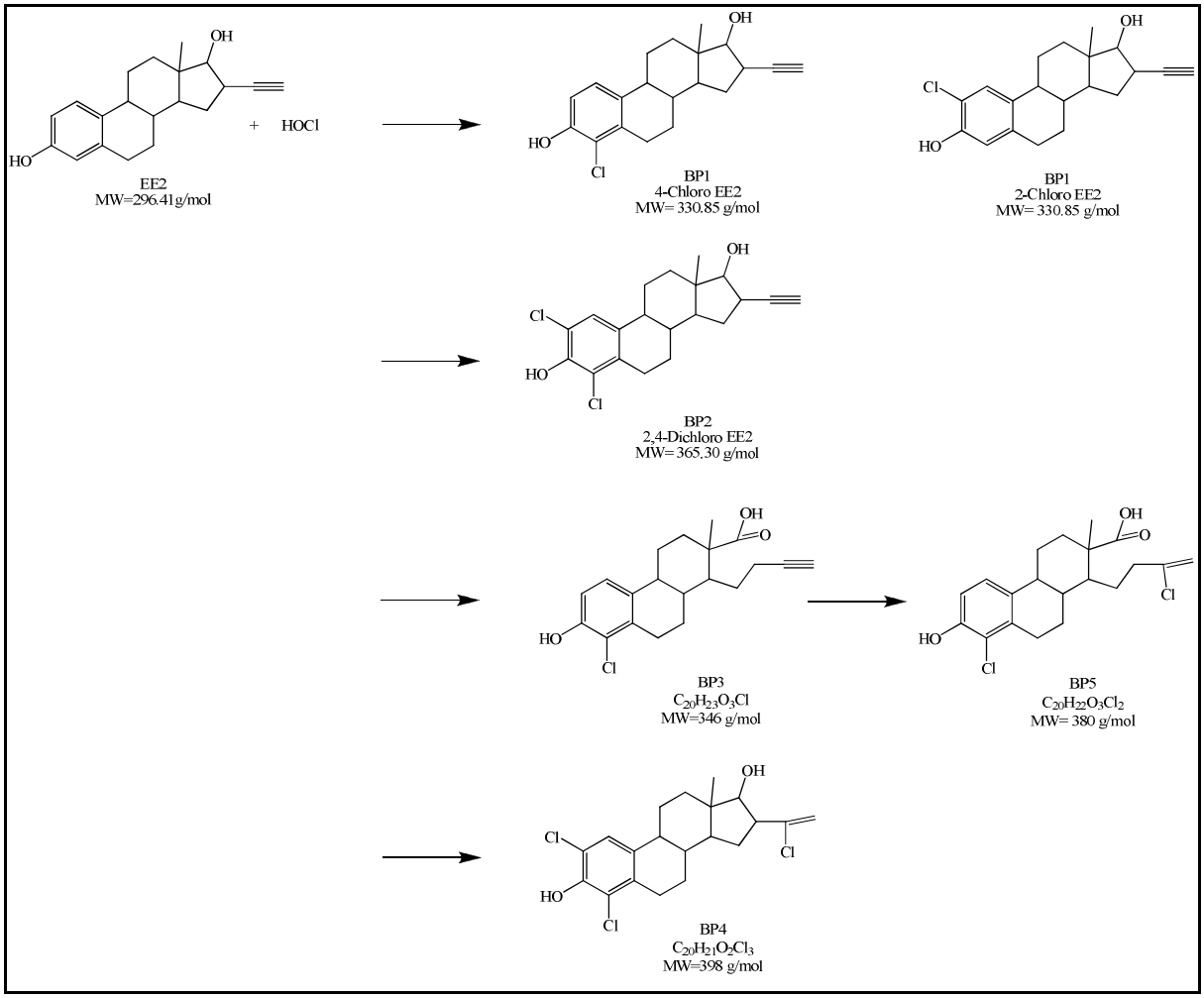


Figure 3-7: Proposed Byproducts of HOCl and EE2

The byproducts found for the chlorination of EE2 are similar to those suggested by Moriyama *et al.* (2003) and Hu *et al.* (2003); however, the byproduct 5 that eluted at 19 min (MW 380 m/z) was not found in any study published on the chlorination of EE2.

Other byproducts are formed from this reaction sequence but were not identified by this method due to their lack of absorbance at 280 nm and there were no other peaks located on the total ion chromatogram. It was determined that other byproducts were formed due to the insufficient sums of EE2 and byproduct peaks areas in comparison to the initial EE2 peak area, assuming EE2 and the byproducts have the same molar absorption coefficients and produced the same mass spectral signal intensity.

Relative Quantification

The relative concentration of each chlorination byproduct was determined based on peak areas since calibration curves were unavailable for each byproduct. For purposes of relative concentrations, it was assumed that the only byproducts formed during the reactions were those that absorbed at a wavelength of 280 nm. The estimated quantification of each byproduct was based on the initial concentration of EE2 (10.0 ± 1.0 μM) and the molecular weights of each byproduct.

For the instantaneous reactions with the chlorine dose of 2.5 mg/L, EE2 produced an 100 % degradation after a reaction time of 1800 sec. For the short-term reactions, the dose of 1.5, 3.0 and 6.0 mg/L Cl_2 and EE2 showed an 82, 97 and 100 % degradation, respectively. For the long-term reactions, doses of 1.5, 3.0 and 6.0 mg/L Cl_2 displayed an 86, 100 and 100 % EE2

degradation, respectively. Figure 3-8 provides the relative concentrations of EE2 and its chlorination byproducts with the corresponding chlorine dose and reaction duration.

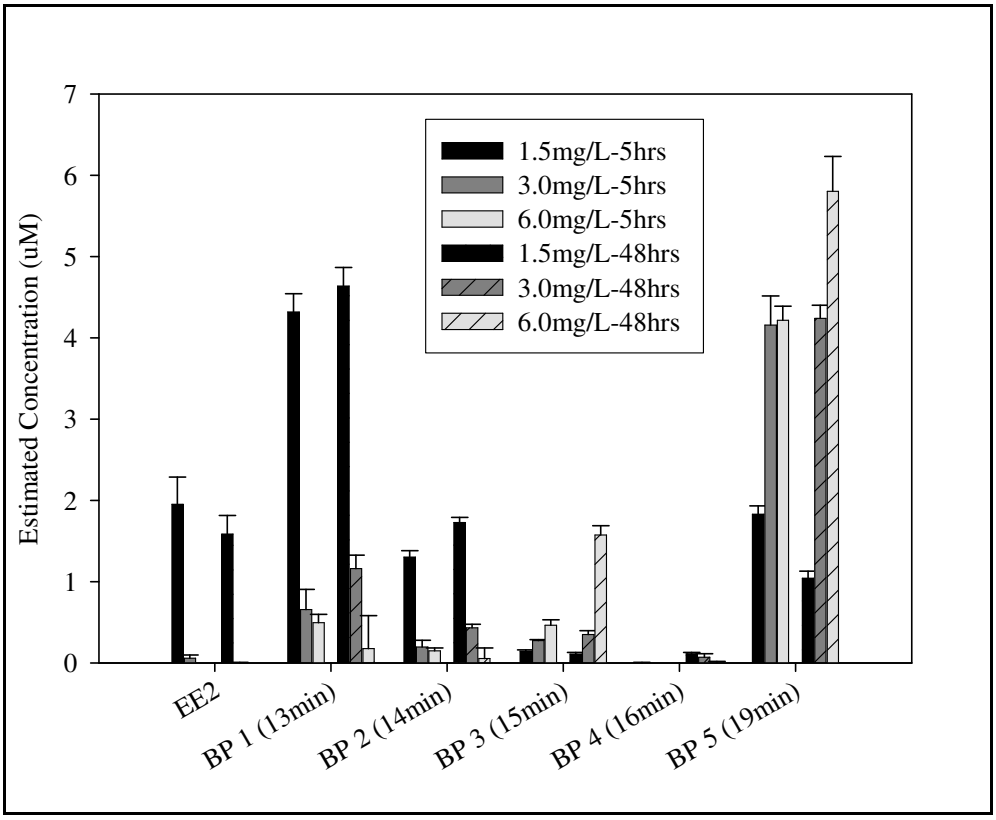


Figure 3-8: Estimated Concentrations of EE2 and Chlorination Byproducts

In order to compare the EE2 concentration and the estimated concentration of the byproducts, it was assumed that the byproducts had the same molar absorption at 280 nm as EE2.

3.5.1.2 Chlorination of DES

Reactions

The byproducts formed during the instantaneous reactions of DES were identical to those observed in the short term and long term reactions. As the dose was increased, the quantity of

the observed byproducts increased, but the number of byproducts remained constant. The byproducts observed were not all immediately formed upon introduction of the chlorine dose, showing various reaction rates and production quantities. Each experiment included initial concentrations of DES of 10.0 ± 1.0 µM and was treated with an excess molar ratio of Cl_2. Each reaction observed, showed a proportional increase in total byproducts with the degradation of DES. Figure 3-9 shows DES and total byproducts results from the instantaneous chlorination of DES.

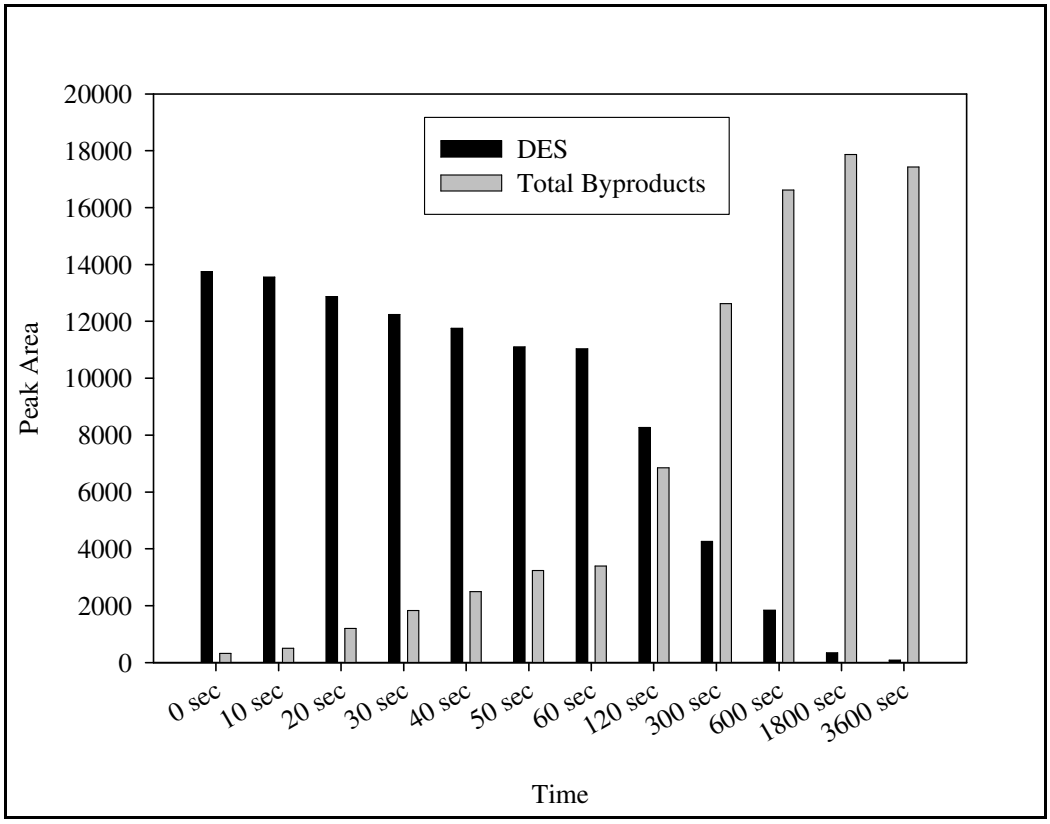


Figure 3-9: Instantaneous Chlorination of Diethylstilbestrol

Chlorination of DES showed a 93 % degradation of the parent compound at 3600 sec reaction time. Each byproduct separated for individual time increments is presented in Appendix B. The chlorine residuals found for the DES long and short term chlorinations are also presented in Appendix B.

Detection

The chlorination of DES followed an expotenial decay function, where the contaminant decreased rapidly in early time intervals and with small changes in concentration after 120 sec of reaction time. At time 3600 sec, DES was detected by LC/MS and it was estimated that 93 % of the initial concentration was degraded. The major byproducts formed from DES and Cl_2 reaction were observed at 13, 14 and 16 min on the chromatogram when the absorbance at 240 nm was isolated. Two minor byproducts were found at 17 and 22 min on the chromatogram; however, there was insufficient information available to determine the precise indentity of these DES and free chlorine byproducts.

According to the the absorbance spectrum for DES, the maximum absorbance wavelength is at 240 nm. The absorbance shows a characteristic phenolic pattern of one local maxima between 190 nm and 400 nm. It was therefore assumed that any byproducts detected having an absorbance at 240 nm and showing a similar absorbance pattern to DES, would still have the parent ring structure intact. Therefore, the PDA was used to isolate specific wavelengths on the total ion chromatogaphs. Figure 3-10 presents the liquid chromatogram with isolated absorbance at 240 nm for the long term chlorination. Instantaneous and short term chlorination chromatograms are found in Appendix B.

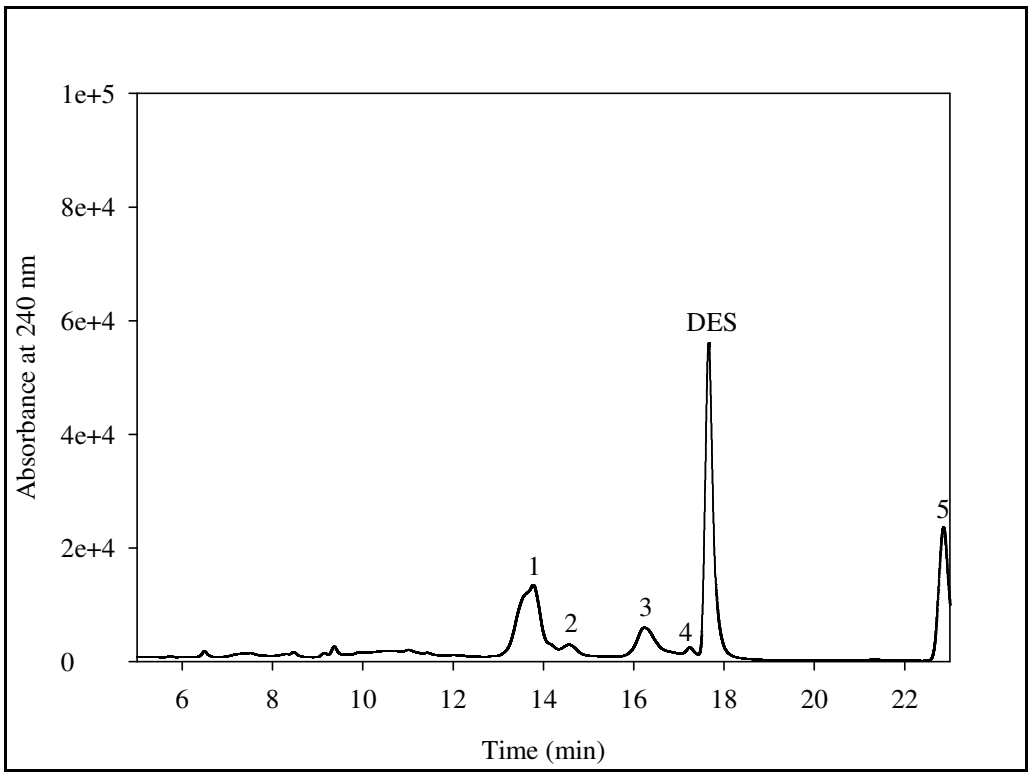


Figure 3-10: Diethylstilbestrol LC Chromatogram with Isolated 240 nm Absorbance

From the isolated chromatogram, each peak was integrated to determine the associated areas. The chlorination reactions produced five byproducts, with the major byproducts 1,2 and 3 eluted at 13, 14 and 16 min, respectively. The peak areas for the three major byproducts and DES are diplayed in Figure 3-11 for the instantaneous chlorination reaction.

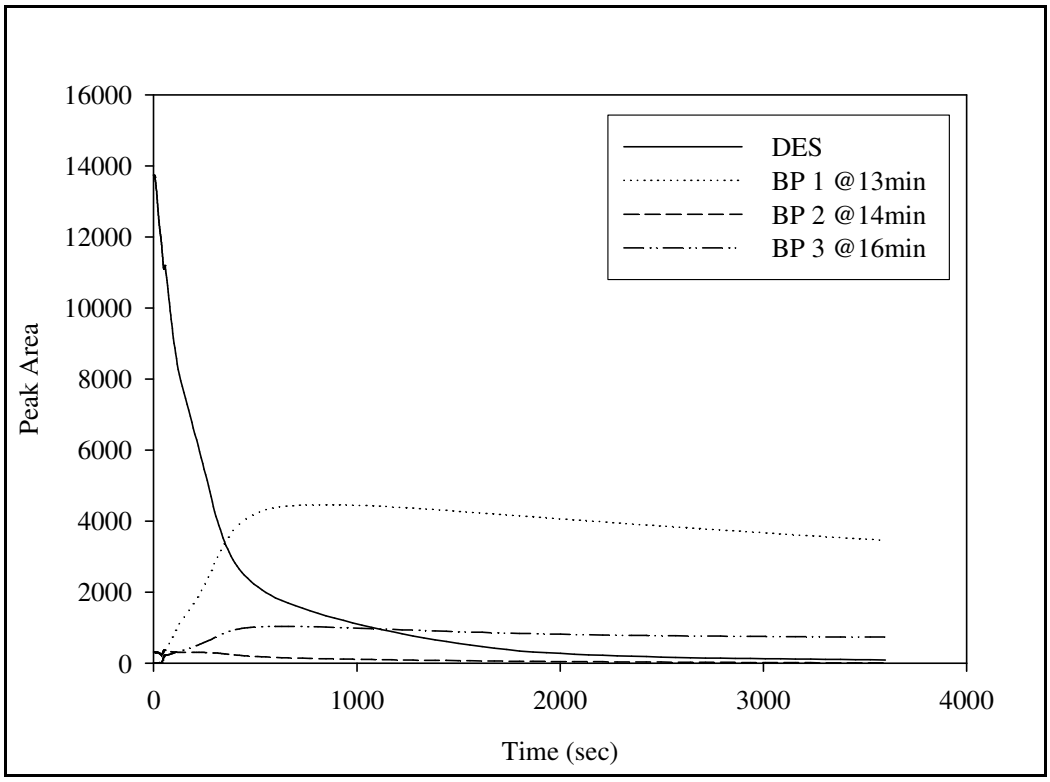


Figure 3-11: Formation of Major Byproducts from Chlorination of Diethylstilbestrol

The reaction between DES and free chlorine did not produce immediate byproducts. All major byproducts began formation after 30 sec of reaction time. The byproducts 1 and 2, found at 13 min and 14 min, reached their maximum concentrations at 600 sec and the byproduct 3 at 16 min reached its maximum quantity at 60 sec.

Identification

The chlorination of DES was performed, finding three dominant byproducts and two minor byproducts with ring structures still intact. The diode array spectrophotometer was used to determine if the byproducts absorbed at 240 nm, which is the relative maximum absorbance of

DES. Figure 3-12 shows the carbon numbering format for DES to help in identification of carbon atoms within each moiety.

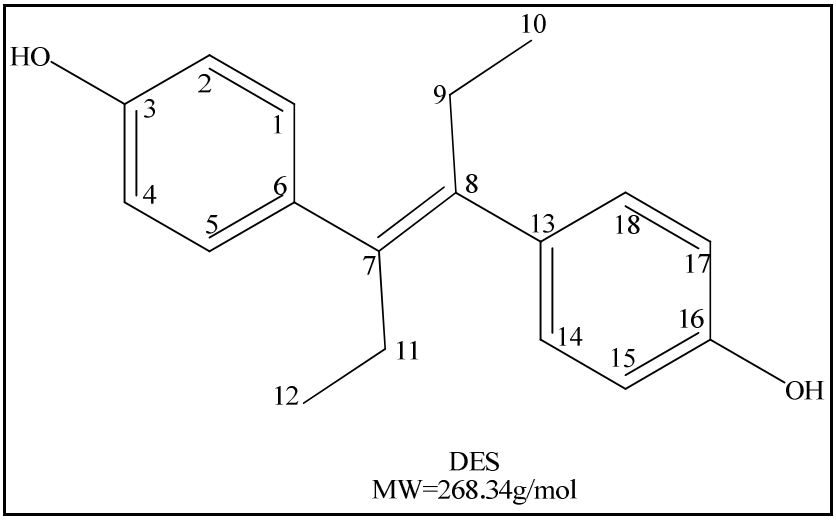


Figure 3-12: Diethylstilbestrol

The byproducts found at 13, 14 and 22 min show the same local maximum pattern as DES. The byproducts at 16 and 17 min contain two local maximum in the corresponding absorbance spectrum. Byproducts at 13, 14, 16 and 17 min elute prior to DES when using a mobile phase of methanol and water and BP 5 at 22 min elutes after DES. The absorbance spectra for DES and the observed chlorination byproducts are shown in Figure 3-13.

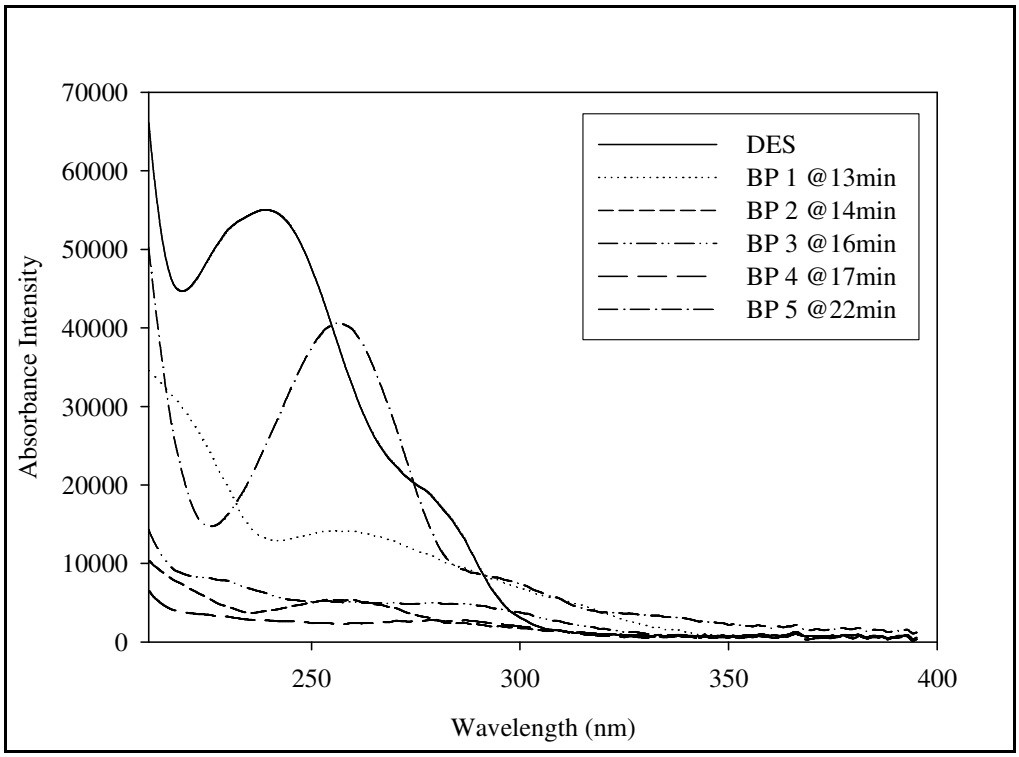


Figure 3-13: Absorbance of DES and Chlorination Byproducts

Based on the retention times with corresponding polarity, mass spectra and absorbance at 240 nm, the major byproducts were identified for the chlorination of DES and are presented in Table 3-3.

Table 3-3: Proposed Identification of DES and Chlorination Byproducts

Analyte	Retention Time (min)	Identity
DES	18 min	DES
Byproduct 1	13 min	Tetrachloro DES (MW 405 m/z)
Byproduct 2	14 min	Monochloro DES (MW 302 m/z)
Byproduct 3	16 min	Trichloro DES (MW 371 m/z)
Byproduct 4	17 min	Dichloro DES (MW 336 m/z)
Byproduct 5	22 min	MW 265 m/z

The proposed reactions between free chlorine and DES are shown in Figure 3-14. There was insufficient information available on byproduct 5 to determine a chemical structure.

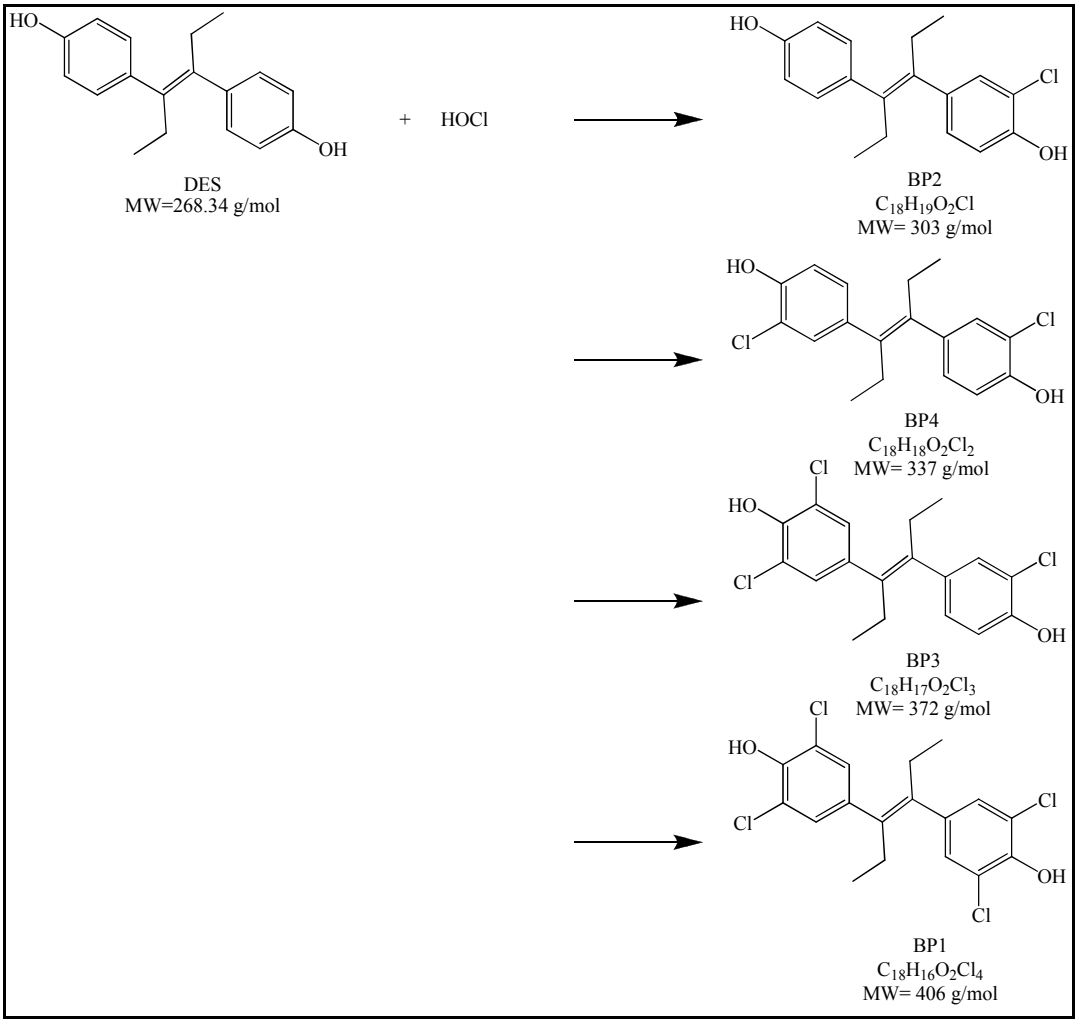


Figure 3-14: Proposed Byproducts of HOCl and DES

The major byproducts, monochloro DES, dichloro DES and tetrachloro DES, were formed approximately 20 sec after the introduction of HOCl into a solution of DES, attacking the both phenolic moieties at the ortho position(s), with respect to the hydroxyl groups at C3 and C16.

The minor byproducts could not be accurately identified due to the lack of molecular ions on the mass spectra. The mass spectra for DES and each byproduct are located in Appendix B.

Relative Quantification

The relative concentration of each chlorination byproduct was determined based on peak areas since calibration curves were unavailable for the respective byproducts. For purposes of relative concentrations, it was assumed that the only byproducts formed during the reactions were those that absorbed at a wavelength of 240 nm. The estimated quantification of each byproduct was based on the initial concentration of DES (10.0 ± 1.0 μM) and the molecular weights found for each byproduct. In order to compare the DES concentration and the estimated concentration of the byproducts, it was assumed that the byproducts had the same molar absorption at 240 nm as DES.

Figure 3-15 provides the relative concentration of chlorination byproducts for both the long and short term reactions. The instantaneous chlorination reactions demonstrated a DES degradation of 93 % at 3600 sec with a dose of 2.5 mg/L Cl_2. For the short-term reactions, the dose included 1.5, 3.0 and 6.0 mg/L Cl_2 and DES displayed a percent degradation of 45, 95 and 97 %, respectively. For the long-term reactions, the dose also included 1.5, 3.0 and 6.0 mg/L Cl_2 and DES showed a 74, 98 and 100 % degradation, respectively.

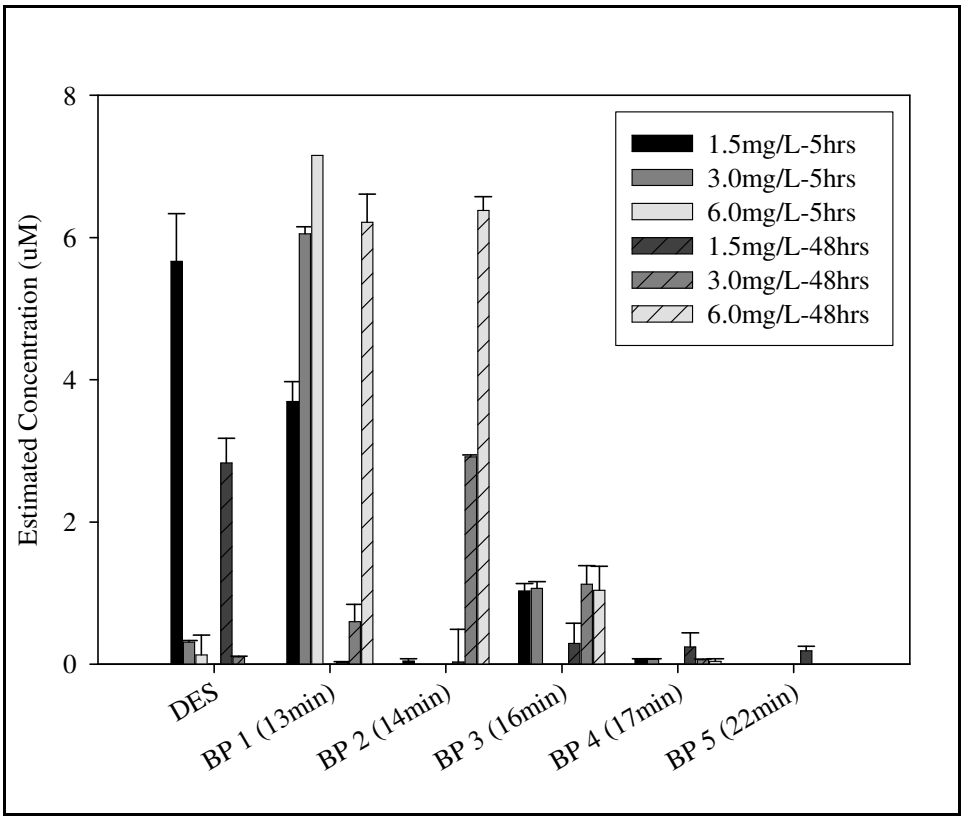


Figure 3-15: Estimated Concentrations of DES and Chlorination Byproducts

3.5.2 Chloramination

Chloramination procedures followed the UMass Standard Operating Procedures. The chloramination disinfection treatment included pre-ammoniation, pre-chlorination, simultaneous addition of ammonia and chlorine solutions and pre-mixed chloramination. The long (48 hrs) and short (5 hrs) contact times for chloramination were preformed using pre-chlorination then ammonia addition as is customary in treatment systems.

3.5.2.1 Chloramination of EE2

Reactions

As the dose was increased, the quantity of the byproducts observed for each chloramination reaction remained constant and the number of byproducts was constant within each method. Each experiment included initial concentrations of EE2 of 10.0 ± 1.0 μM and was treated with a molar ratio of Cl_2:N of 4 and a Cl_2 concentration of 2.5 mg/L.

For the pre-ammoniation chloramination reaction with EE2, there was one byproduct formed. The simultaneous addition of chlorine and ammonia created two byproducts from the resulting reaction. With the pre-formed chloramines solution, there were no byproducts that formed during the reaction with EE2.

In the pre-chlorination instantaneous reactions, there were two byproducts produced that were the same as those produced in the short-term reaction. The byproducts formed during the pre-chlorination chloramination instantaneous reactions of EE2 were identical to those observed in the pre-chlorination long term reactions. As observed in each type of chloramination reaction with EE2, the addition of ammonia to the solution affectively quenched the free chlorine present and significantly decreased the reaction rates.

Each byproduct separated for individual time increments is presented in Appendix B, along with the total byproducts and EE2 peak areas for the instantaneous chloramination reactions. The chlorine residuals found for the EE2 long and short term chloraminations are also available in Appendix B.

Detection

EE2 showed minute degradation rates associated with each chloramination method. The byproduct formed from EE2 and pre-ammoniation chloramination reactions was observed at 13 min elution time on the chromatogram based on absorbance at 280 nm was isolated. Two byproducts were found during the pre-chlorination and simultaneous addition instantaneous reactions and were observed at 13 and 19 min on the chromatograms when the absorbance at 280 nm was isolated. The pre-formed chloramine chloramination chromatogram did not produce byproducts that absorbed at 280 nm.

For the short-term, pre-chlorination chloramination reactions, there were two byproducts formed at 13 and 19 min. The same byproducts were formed, eluting at 13 and 19 min, as a result of long-term, pre-chlorination chloramination reactions.

Figure 3-16 displays the liquid chromatograms for chloramination reactions with isolated absorbance at 280 nm.

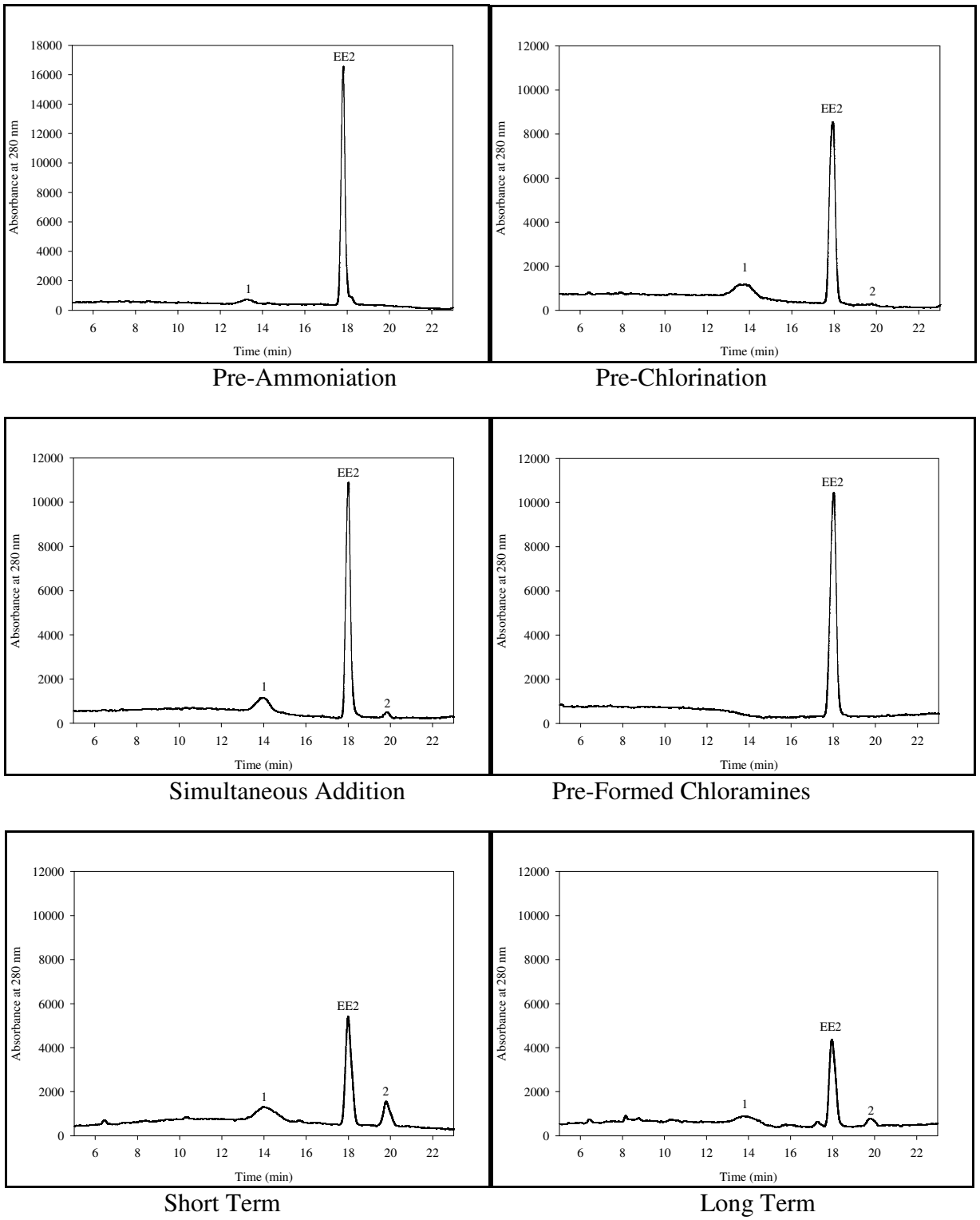


Figure 3-16: Chloramination of Ethinylestradiol; LC Chromatograms Based on Absorbance at 280 nm

Table 3-4 provides the percent degradation and number of byproducts formed for each respective chloramination type and chloramine dose.

Table 3-4: Chloramination of EE2

Chloramination Type	Dose (Cl_2/N of 4)	% Degradation of EE2	Number of Byproducts*
Pre-Ammoniation (3600 sec)	2.5 mg/L Cl_2	9 %	1
Pre-Chlorination (3600 sec)	2.5 mg/L Cl_2	27 %	2
Simultaneous Addition (3600 sec)	2.5 mg/L Cl_2	9.5 %	2
Pre-Mixed Chloramination (3600 sec)	2.5 mg/L Cl_2	3 %	0
Short Term Pre-Chlorination	1.5 mg/L Cl_2	19 %	2
	3.0 mg/L Cl_2	35 %	2
	6.0 mg/L Cl_2	52 %	2
Long Term Pre-Chlorination	1.5 mg/L Cl_2	28 %	2
	3.0 mg/L Cl_2	50.5 %	2
	6.0 mg/L Cl_2	61.5 %	2

*Byproducts produced from treatment with substantial absorbance at 280 nm

Identification

The chloramination of EE2 was performed, finding two dominant byproducts with ring structures intact. The diode array spectrophotometer was used to determine if the byproducts absorbed at 280 nm, which was the relative maximum absorbance of EE2. Figure 3-5 shows the carbon numbering format for EE2 to help in identification of carbon atoms within each moiety.

The byproduct 1 found at 13 min conforms to the same two local maxima pattern as EE2 and elutes prior to EE2 when using a mobile phase of methanol and water. The byproduct 2 at 19 min contains only one local maximum in the corresponding absorbance spectrum and elutes after EE2. The absorbance spectra for EE2 and the observed chloramination byproducts are presented in Figure 3-17.

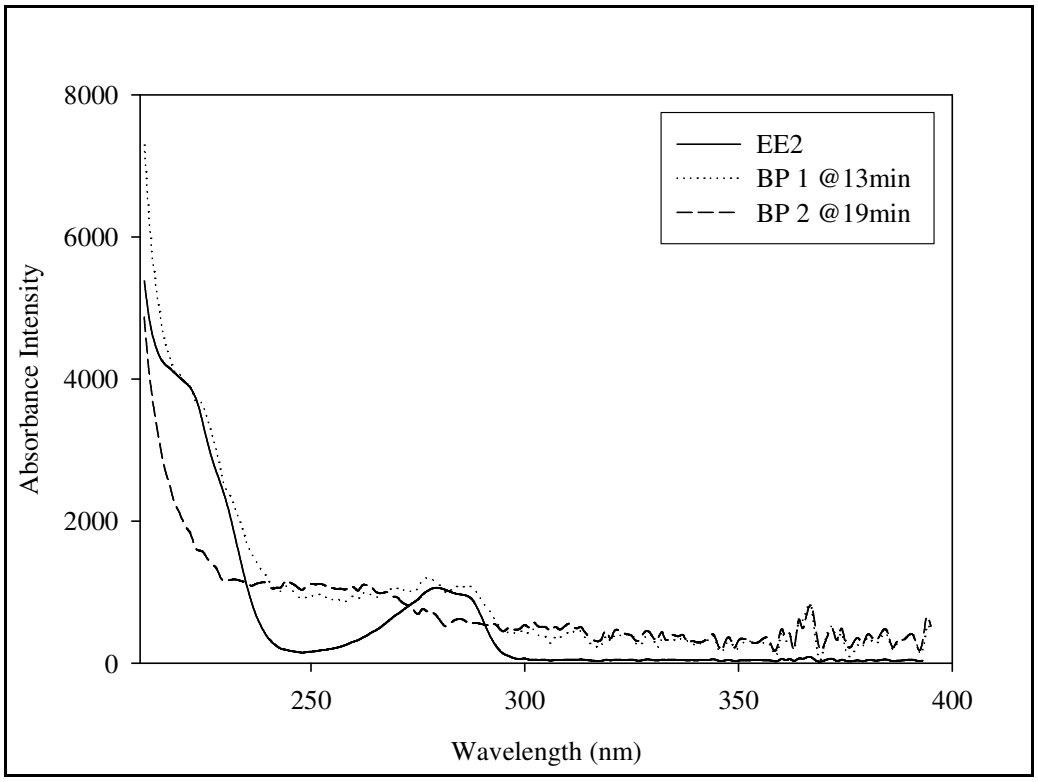


Figure 3-17: Absorbance of EE2 and Chloramination Byproducts

Based on the retention times and corresponding polarity, mass spectra and absorbance at 280 nm, the major byproducts were identified for the chloramination of EE2 and are presented in Table 3-5.

Table 3-5: Proposed Identification of EE2 and Chloramination Byproducts

Analyte	Retention Time (min)	Identity
EE2	17 min	Ethinylestradiol
Byproduct 1	13 min	$C_{20}H_{21}O_2Cl_3$ MW 398 m/z
Byproduct 2	19 min	$C_{20}H_{22}O_3Cl_2$ MW 380 m/z

The major byproducts, MW 398 m/z and MW 379 m/z, were formed during the long-term chloraminations with 3.0 and 6.0 mg/L chloramines doses. The byproduct 1 at 13 min (MW 398 m/z) was created by the free-chlorine attacking the phenolic moiety at the ortho positions, with respect to the hydroxyl group at C3, and C18. Free-chlorine was available for the reaction with EE2 due to the pre-chlorination step within the chloramination type.

The byproduct 2 at 19 min (MW 379 m/z) was created by the free-chlorine attacking the phenolic moiety at the ortho position (C4 or C2) and losing a hydrogen atom to produce a carbonyl group replacing the hydroxyl group at C3. The five carbon ring opened to form a carboxylic acid group on C17. An addition of a chlorine atom to C18 resulted in the change of the triple bond to a double bond from C18 to C19.

The mass spectra for EE2 and each chloramination byproduct formed are found in Appendix B. The proposed reactions between chloramines and EE2 are shown in Figure 3-18.

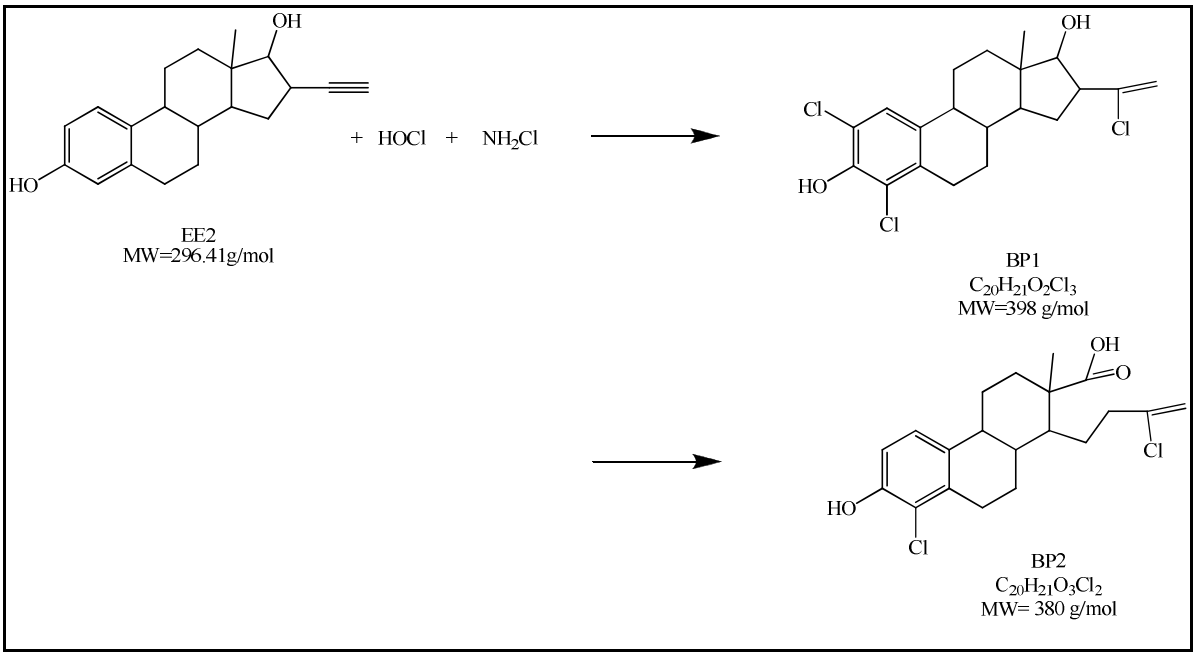


Figure 3-18: Proposed Byproducts of NH_2Cl and EE2

The chloramination byproducts generally conform to the same structures as that of the chlorination byproducts unless amine reactive functional groups are present (Bull and Kopfler, 1991). Since EE2 does not contain moieties that are reactive with amines but are highly reactive with free chlorine, the byproducts that formed as a result of each type of chloramination were the same as the chlorination byproducts. The major differences between the chlorination byproducts and the chloramination byproducts for EE2 reactions were the slower reaction rates and decreased quantity of byproduct formation for the chloramination reactions.

Relative Quantification

The relative concentration of each chloramination byproduct was determined based on peak areas since authentic standards were unavailable for the proposed byproduct. For purposes of relative concentrations, it was assumed that the only byproducts formed during the reactions

were those that absorbed at a wavelength of 280 nm. The estimated quantification of each byproduct was based on the initial concentration of EE2 (10.0 ± 1.0 µM) and the molecular weights determined for each byproduct.

Figure 3-19 provides the relative concentrations of EE2 and its chloramination byproducts with respect to the dose and reaction time.

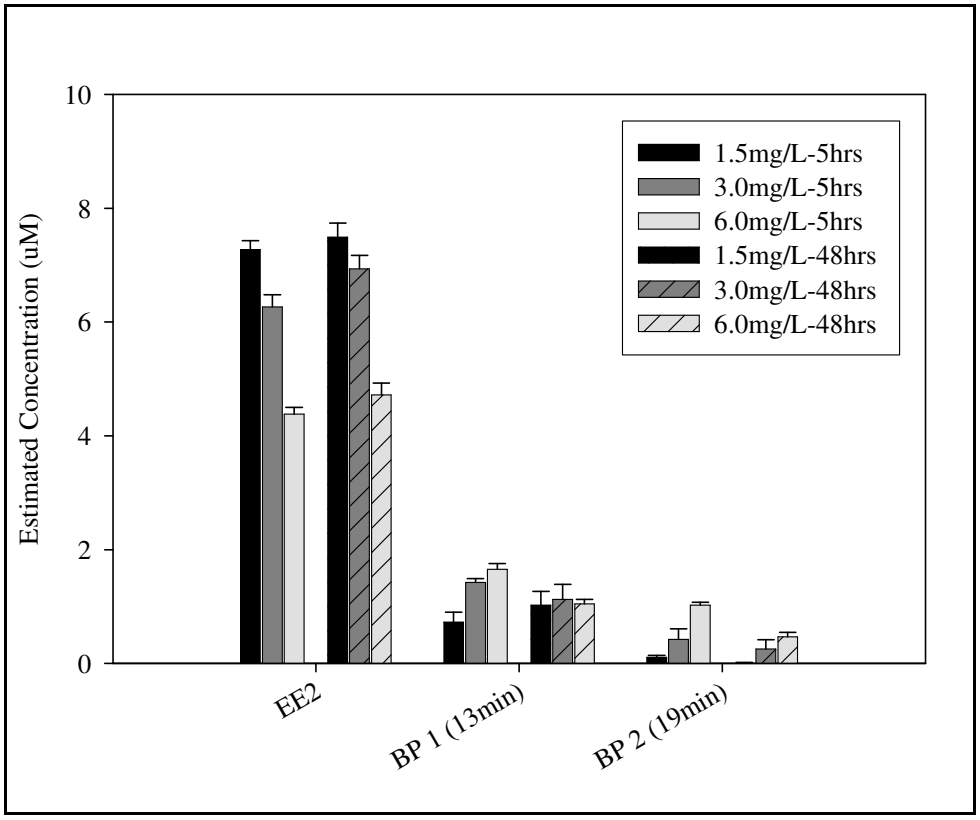


Figure 3-19: Estimated Concentrations of EE2 and Chloramination Byproducts

The instantaneous chloramination reactions displayed an EE2 degradation of 9, 27, 9.5 and 3 % at 3600 sec with a dose of 2.5 mg/L Cl_2 and a Cl_2:N ratio of 4, for pre-ammoniation, pre-chlorination, simultaneous addition and pre-formed addition respectively. For the short-term

pre-chlorination reactions, the dose included 1.5, 3.0 and 6.0 mg/L Cl_2 with a Cl_2:N ratio of 4 and EE2 showed a percent degradation of 19, 35 and 52 %, respectively. For the long-term pre-chlorination reactions, the dose also included 1.5, 3.0 and 6.0 mg/L Cl_2 with and a Cl_2:N ratio of 4 and EE2 demonstrated a 28, 50.5 and 61.5 % degradation, respectively.

3.5.2.2 Chloramination of DES

Reactions

As the dose was increased, the quantity of the byproducts observed for each chloramination reaction remained constant and the number of byproducts stayed constant within each method. Each experiment included initial concentrations of DES of 10.0 ± 1.0 µM and was treated with a molar ratio of Cl_2:N of 4 and a Cl_2 concentration of 2.5 mg/L.

For the pre-ammoniation, the simultaneous addition of chlorine and ammonia and the pre-formed chloramines solution reactions, no byproducts formed during the reactions with DES. In the pre-chlorination instantaneous reactions, there were also no byproducts produced. The byproducts formed during the pre-chlorination chloramination short term reactions of DES were identical to those observed in the pre-chlorination long term reactions; however, there was an additional byproduct detected for the long-term reaction. As observed in each type of chloramination reaction with DES, the addition of ammonia to the solution affectively quenched the free chlorine present and significantly decreased the reaction rates.

Each byproduct separated for individual time increments is presented in Appendix B, along with the total byproducts and DES peak areas for the instantaneous chloramination reactions. The

chlorine residuals found for the DES long and short term chloraminations are also presented in Appendix B.

Detection

DES showed small degradation rates associated with each chloramination method. The byproducts formed from DES and the chloramination reactions were not observed on the chromatogram when the absorbance at 280 nm was isolated. For the short-term, pre-chlorination chloramination reactions, there were two byproducts formed with retention times of 14 and 17 min. Three byproducts were formed with retention times of 14, 15 and 17 min as a result of long-term, pre-chlorination chloramination reactions.

Figure 3-20 shows the liquid chromatograms for chloramination reactions with isolated absorbance at 240 nm.

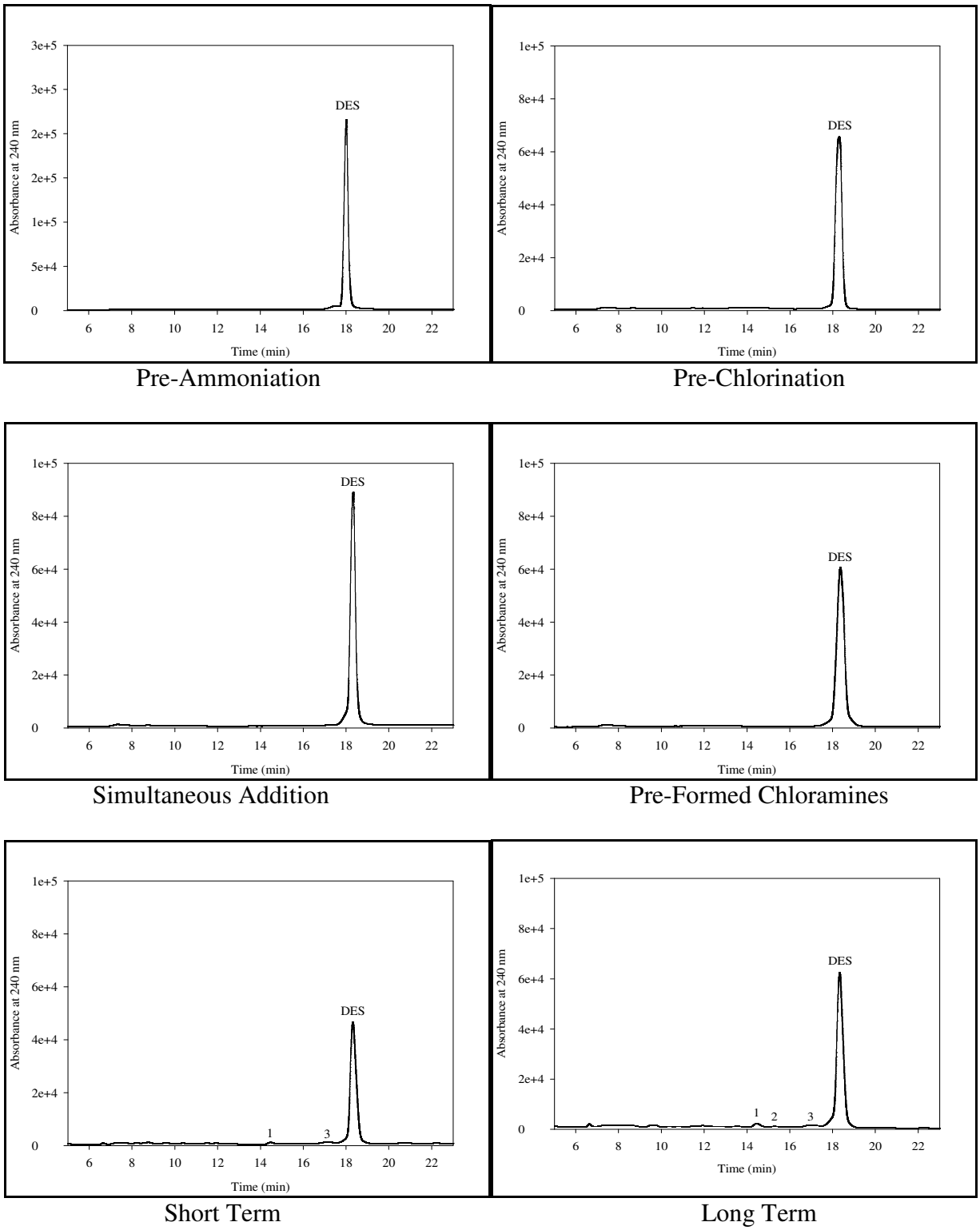


Figure 3-20: Chloramination of Diethylstilbestrol; LC Chromatograms Based on Absorbance at 240 nm

Table 3-6 provides the resulting percent degradation and number of byproducts formed for each respective chloramination type and chloramine dose.

Table 3-6: Chloramination of DES

Chloramination Type	Dose (Cl_2/N of 4)	% Degradation of DES	Number of Byproducts*
Pre-Ammoniation	2.5 mg/L Cl_2	8 %	0
Pre-Chlorination	2.5 mg/L Cl_2	18 %	0
Simultaneous Addition	2.5 mg/L Cl_2	7 %	0
Pre-Mixed Chloramination	2.5 mg/L Cl_2	1 %	0
Short Term Pre-Chlorination	1.5 mg/L Cl_2	7 %	1
	3.0 mg/L Cl_2	16 %	2
	6.0 mg/L Cl_2	37 %	2
Long Term Pre-Chlorination	1.5 mg/L Cl_2	20.5 %	1
	3.0 mg/L Cl_2	33 %	3
	6.0 mg/L Cl_2	55 %	3

*Byproducts produced from treatment with substantial absorbance at 240 nm.

The reaction between DES and chloramines did not produce immediate byproducts. Major byproduct formation began during the short term reaction time. The three major byproducts resulting from the long and short term chloraminations remained constant during the 3.0 and 6.0 mg/L dose. At the 1.5 mg/L dose, however, only one byproduct was produced, which was the same compound for both short and long reaction times.

Identification

The chloramination of DES was performed, finding three dominant byproducts with ring structures still intact. The diode array spectrophotometer was used to determine whether the byproducts absorbed at 240 nm, which is the relative maximum absorbance of DES. Figure 3-12 presents the carbon numbering format for DES to assist in identification of carbon atoms within each moiety.

The byproducts found at 14, 15 and 17 min show the same local maximum pattern as DES. The chloramination byproducts at 14, 15 and 17 min elute prior to DES when using a mobile phase of methanol and water. The absorbance spectra for DES and the observed chlorination byproducts are shown in Figure 3-21.

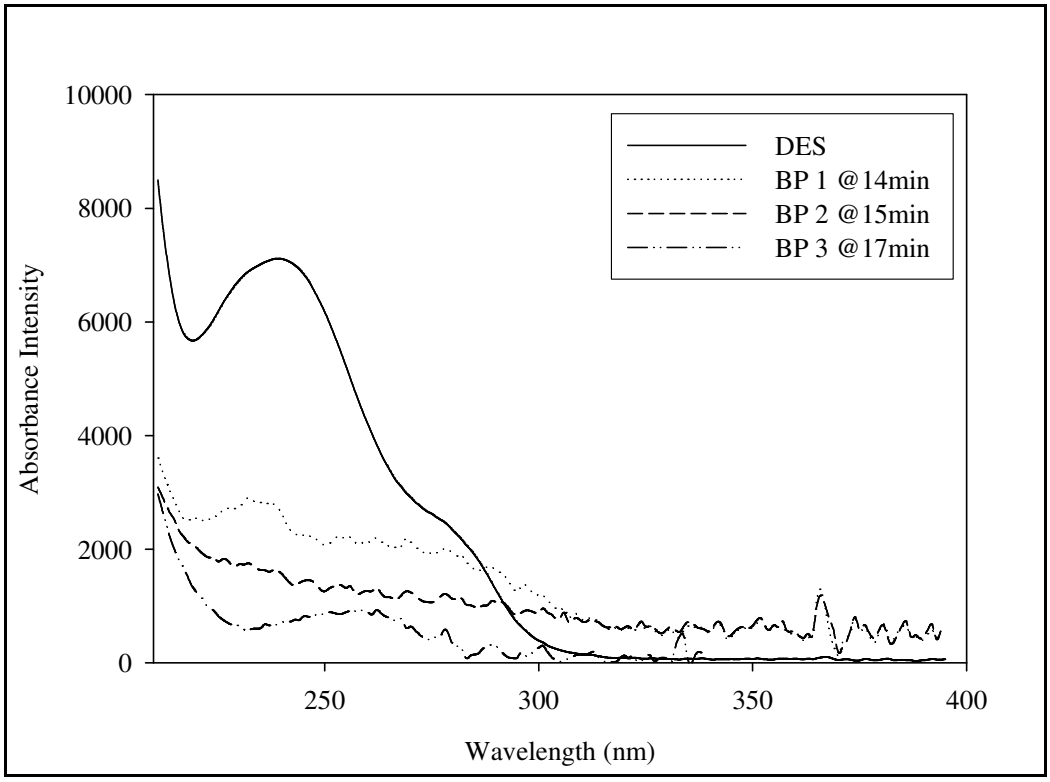


Figure 3-21: Absorbance of DES and Chloramination Byproducts

Based on the retention times and corresponding polarity, mass spectra and absorbance at 240 nm, the major byproducts were identified for the chloramination of DES and presented in Table 3-7.

Table 3-7: Proposed Identification of DES and Chloramination Byproducts

Analyte	Retention Time (min)	Identity
DES	18 min	Diethylstilbestrol
Byproduct 1	14 min	Dichloro DES MW 339 m/z
Byproduct 2	15 min	Monochloro DES MW 302 m/z
Byproduct 3	17 min	Monochloro DES MW 302 m/z

Three major byproducts, monochloro DES, dichloro DES and trichloro DES, were formed during the long-term pre-chlorination chloramination of DES. These byproduct formations were the result of free-chlorine attacking both phenolic moieties at the ortho position(s), with respect to the hydroxyl groups at C3 and C16.

The mass spectra for DES and each chloramination byproduct formed are located in Appendix B. The proposed reactions between chloramines and DES are shown in Figure 3-22.

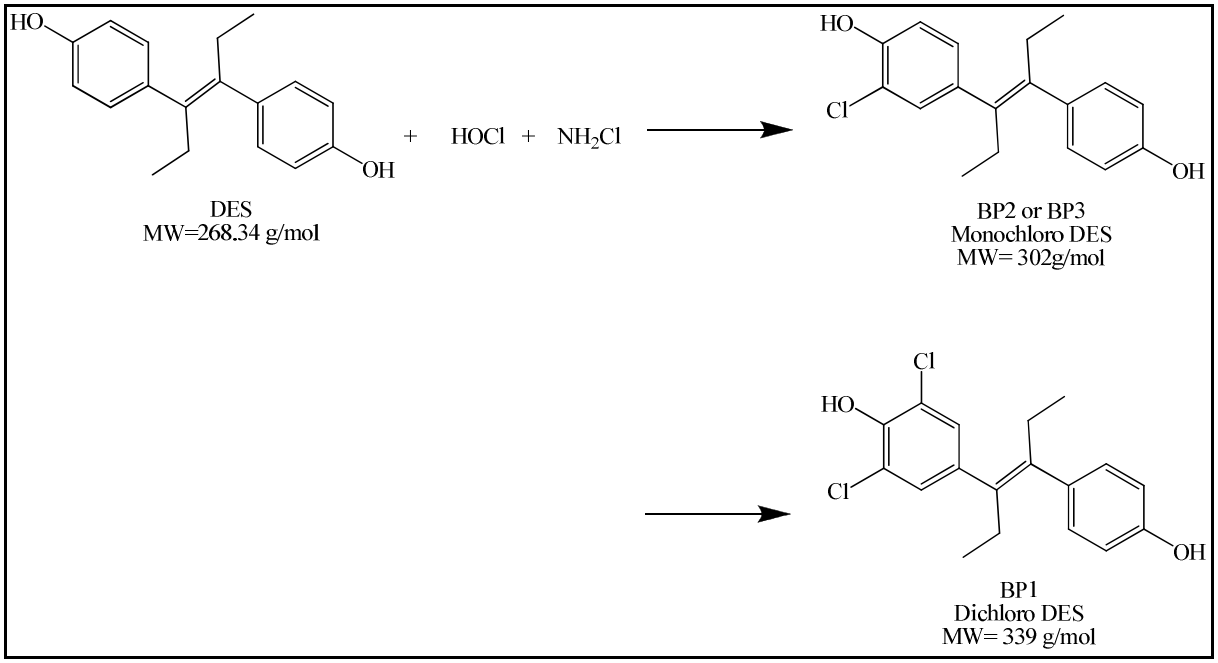


Figure 3-22: Proposed Byproducts of NH_2Cl and DES

As seen with the chloramination of EE2, the byproducts of DES chloramination were similar to the chlorination byproducts; however, the chlorination reactions produced fewer numbers and lesser quantities of byproducts. The byproducts which were formed in the chloramination of DES were the result of free chlorine reacting with DES instead of chloramines reacting with DES.

Due to the apparent polarity of the byproduct 1 at 14 min, as observed on the chromatogram, and the molecular weight, as identified on the mass spectrum, this byproduct was identified as dichloro DES, with both chlorine atoms on the same phenolic moiety, which produced a relatively high polarity. The byproducts that eluted at 15 and 17 min showed identical molecular weights and fragmentation patterns; therefore, it was assumed that both byproducts were monochloro DES with the chlorine atom attaching at different positions on the parent compound.

Relative Quantification

The relative concentration of each chloramination byproduct was determined based on peak areas since calibration curves were unavailable for individual byproducts. For purposes of relative concentrations, it was assumed that the only byproducts formed during the reactions were those that absorbed at a wavelength of 240 nm. The estimated quantification of each byproduct was based on the initial concentration of DES (10.0 ± 1.0 μM) and the molecular weights found for each byproduct. Figure 3-23 presents DES and its chloramination byproducts with respect to dose and reaction time.

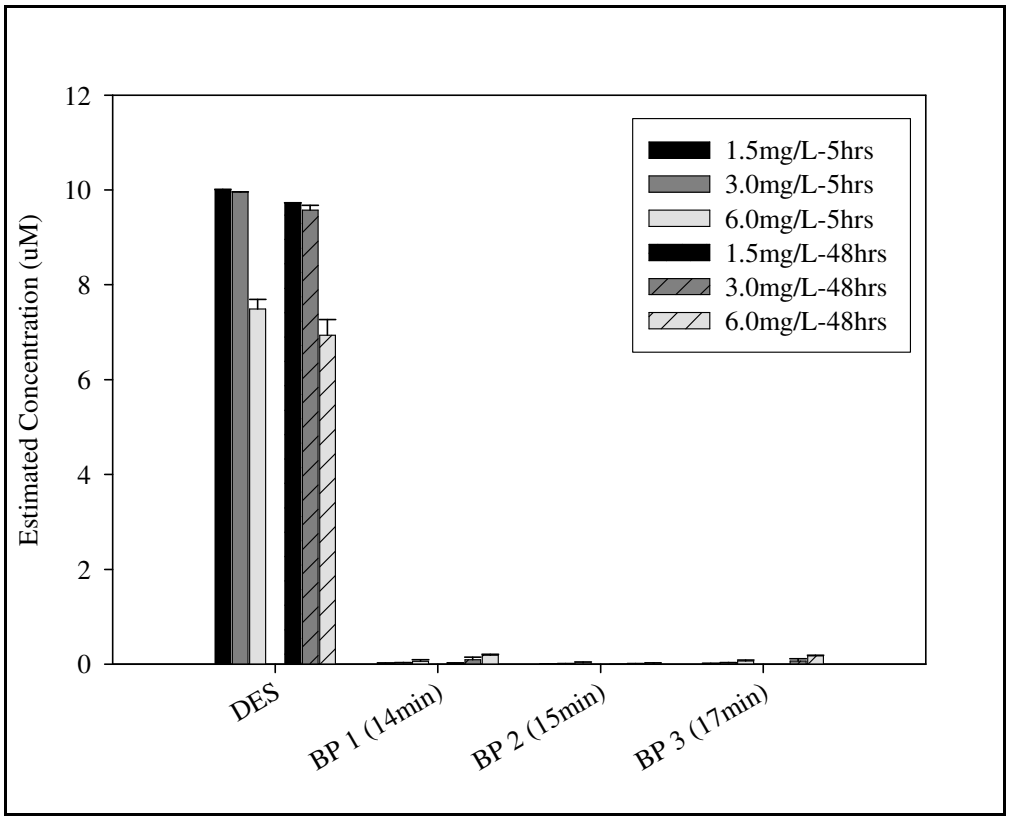


Figure 3-23: Estimated Concentrations of DES and Chloramination Byproducts

The instantaneous chloramination reactions produced a DES degradation of 8, 18, 7 and 1 % at 3600 sec with a dose of 2.5 mg/L Cl_2 and a Cl_2:N ratio of 4, for pre-ammoniation, pre-chlorination, simultaneous addition and pre-formed addition respectively. For the short-term reactions, the dose included 1.5, 3.0 and 6.0 mg/L Cl_2 with a Cl_2:N ratio of 4 and DES demonstrated a percent degradation of 7, 16 and 37 %, respectively. For the long-term reactions, the dose also included 1.5, 3.0 and 6.0 mg/L Cl_2 with and a Cl_2:N ratio of 4 and DES established a 20.5, 33 and 55% degradation, respectively.

3.5.3 Ozonation

Ozonation procedures followed the UMass Standard Operating Procedures, with specific experimental procedures presented in Appendix B. Ozone concentrations were determined using absorbance of O_3 at 258 nm and the molar absorption coefficient (ε_{258nm} = 3000 $M^{-1}cm^{-1}$). Due to the possible functional groups added to the byproducts, both ESI negative and ESI positive modes were implemented on the LC/MS, which provided the ability to attach corresponding charges in the different ionization modes. The data for the different ESI modes are presented separately due to the differences in signal intensity within the different modes and the resulting impact on the relative byproduct formation potentials. When the relative concentration of the byproducts are presented, only byproducts that absorb at the corresponding parent compound local maximum (280 nm or 240 nm) were included, which denotes the intact ring structure. Since the relative concentrations are based on peak areas and the original parent concentration, both positive and negative ionization modes data were separated to determine the relative concentrations for each byproduct with the ring structure intact.

The instantaneous ozonation reactions were not based on time intervals but were selected on a molar ratio of ≤ 1 as compared to the respective EDC contaminant. With an initial concentration of 10 ± 0.1 μM, the concentrations of O_3 selected were as follows: 1, 2, 4, 6, 8 and 10 μM. The short term ozonation reactions were also based on concentration, which were selected on molar ratios of ≥ 1 as compared to the respective EDC contaminant. The short term ozonation concentrations were 1.5, 2.0, 3.0 and 6.0 mg/L O_3.

Ozone reaction durations vary with the amount of ozone added to a solution and the amount of contaminant in the solution; therefore, it was assumed that the ozonation reaction would be complete after 10 min of reaction time. This significantly greater period of time, as compared to the reaction rates determined for the ozonation of EE2, was selected to meet the assumption that the reaction between the parent and/or the byproduct was complete with regards to the ozone reaction (Deborde *et al.*, 2005).

Ice baths were used to increase the concentration of ozone in stock solutions. This decrease in temperature to approximately 5 ^{o}C, interfered with the absorbance measurements, due to the condensation forming on the cuvette, which in turn produced artificially high absorbance values. The lower temperature of the ozone stock solution in comparison to the sample solution may also have decreased the reaction rates.

3.5.3.1 Ozonation of EE2

Reactions

Figure 3-24 and Figure 3-26 show EE2 and total byproducts peak areas versus ozone dose, which resulted from the instantaneous and short term ozonation of EE2 for ESI negative, respectively. Figure 3-25 and Figure 3-27 show EE2 and total byproducts peak areas versus ozone dose, which resulted from the instantaneous and short term ozonation of EE2 for ESI positive, respectively. Each experiment included initial concentrations of EE2 of 10.0 ± 1.0 µM.

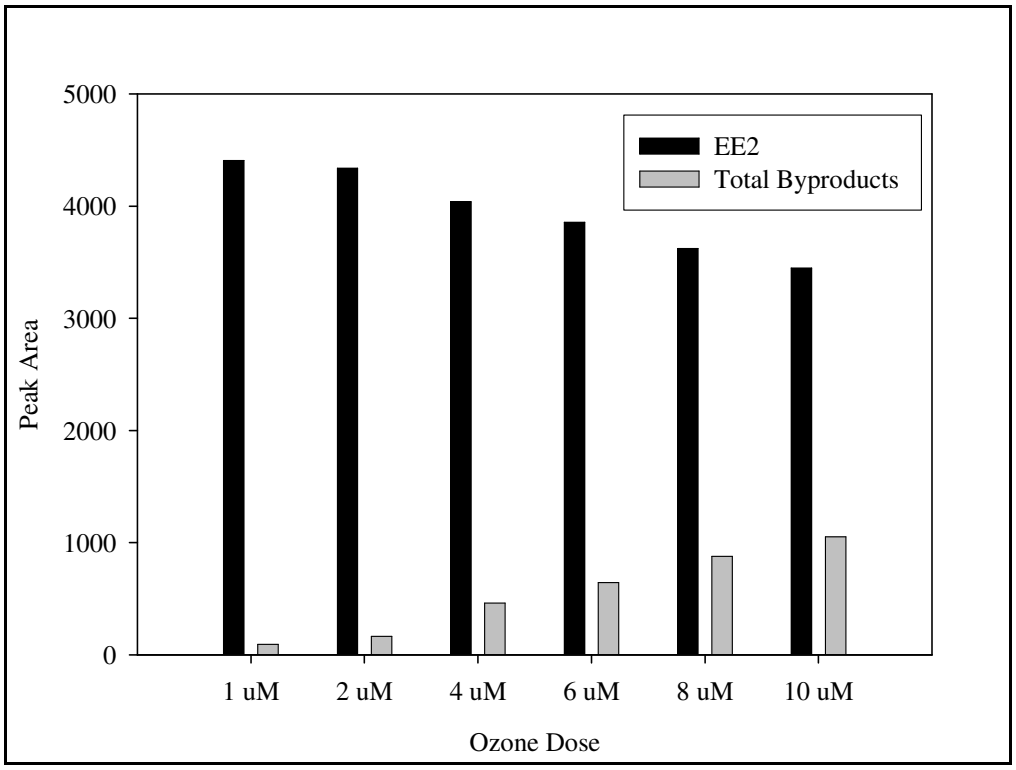


Figure 3-24: Instantaneous Ozonation of EE2 (ESI Negative)

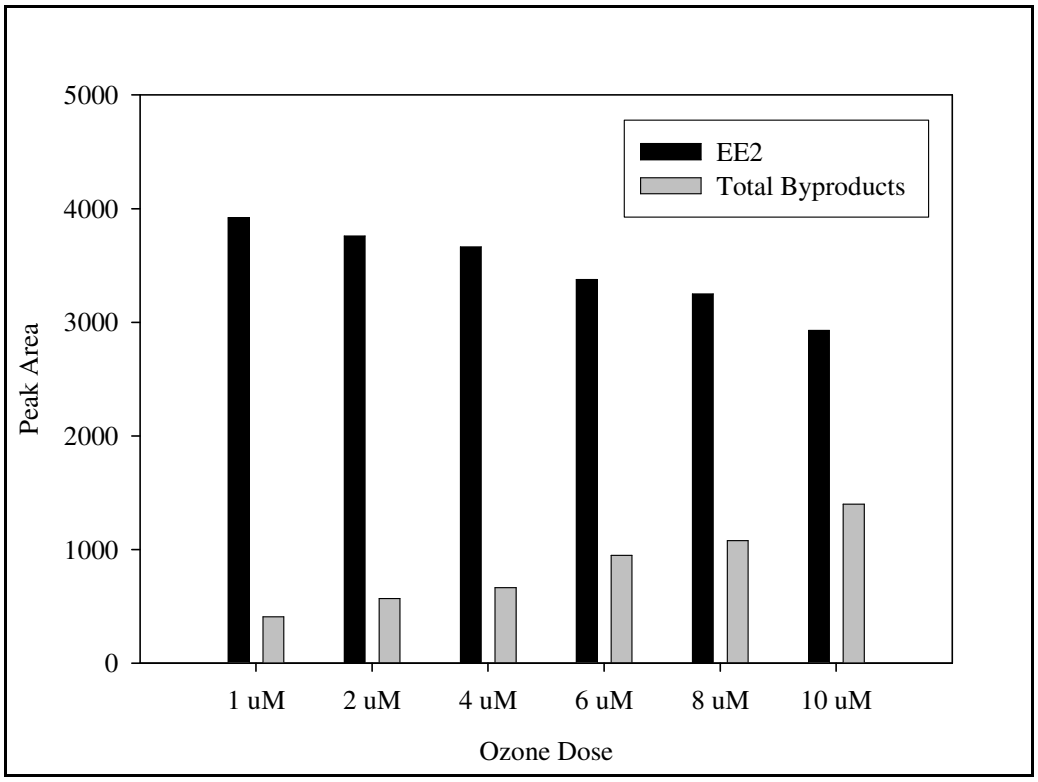


Figure 3-25: Instantaneous Ozonation of EE2 (ESI Positive)

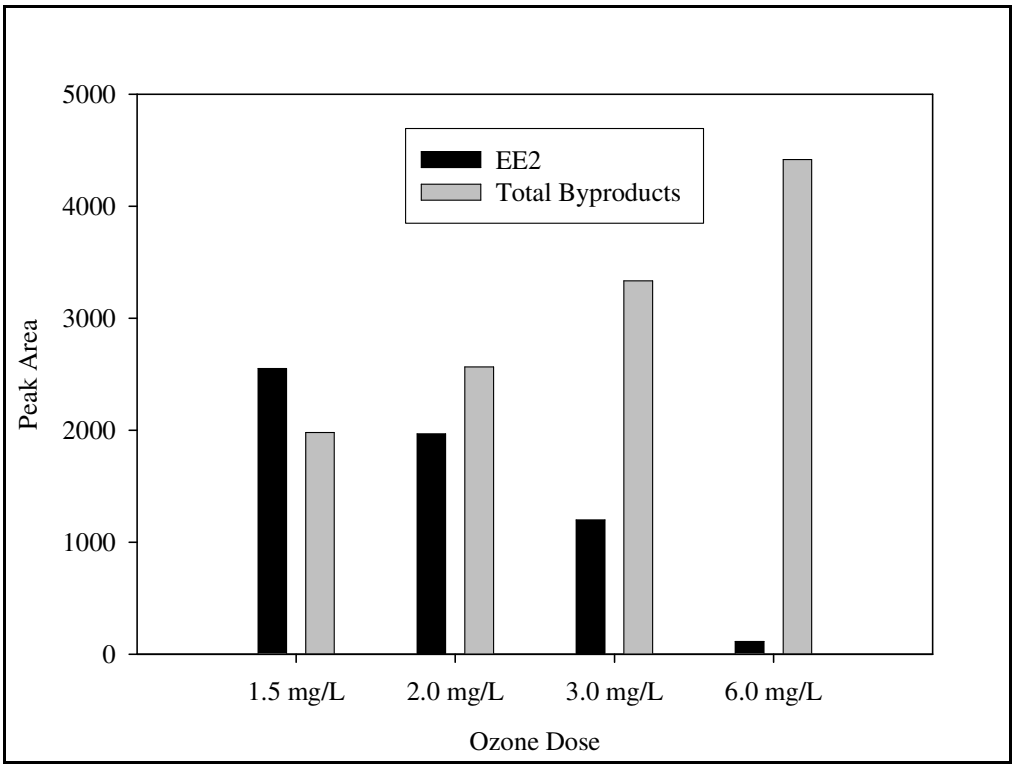


Figure 3-26: Short Term Ozonation of EE2 (ESI Negative)

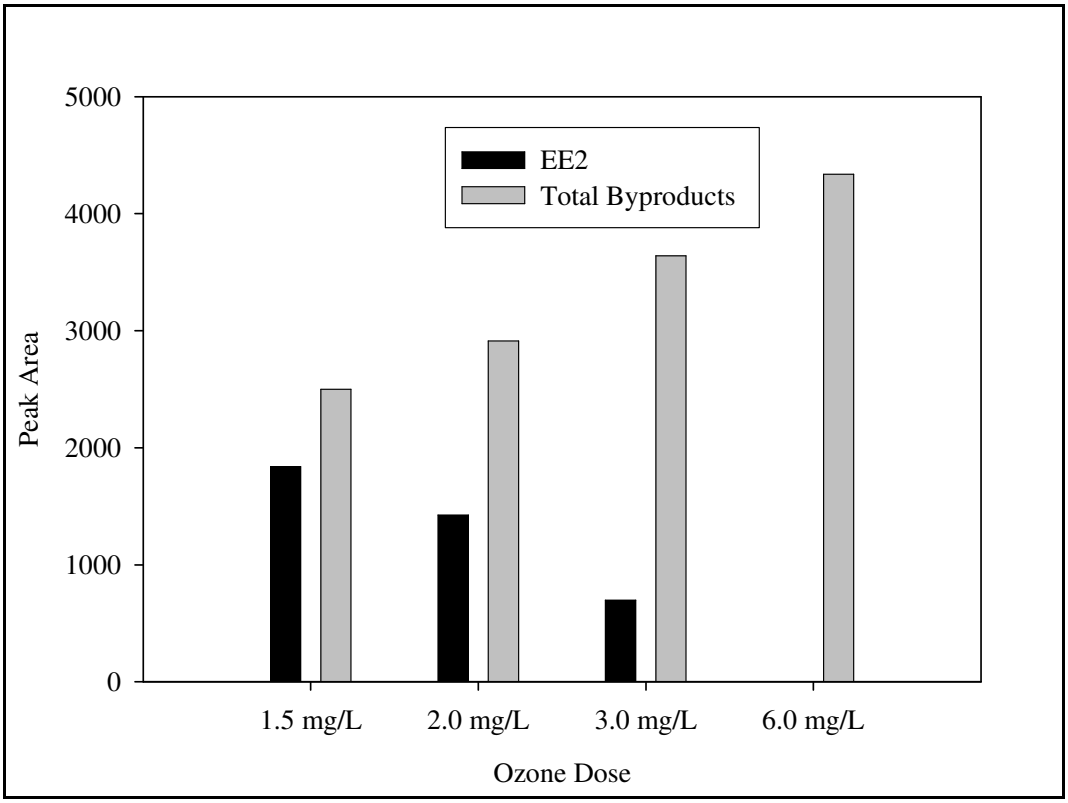


Figure 3-27: Short Term Ozonation of EE2 (ESI Positive)

For each ozonation reaction, it was observed that as EE2 was degraded, the total peak area of the byproducts increased. As the ozone dose was increased, the EE2 concentration also decreased in similar proportions to the O_3 added for the disinfection reaction. Results for each byproduct separated for individual ozone doses is presented in Appendix B for both the instantaneous and short term ozonation of EE2.

Detection

EE2 displayed a linear decay function for both the instantaneous and short term ozonation reactions. The byproducts formed from EE2 and instantaneous O_3 reaction were observed at 10 and 13 min on the chromatogram when the absorbance at 280 nm was isolated in ESI negative mode. At an O_3 dose of 10 μM, 23 % of the EE2 concentration degraded.

Figure 3-28 and Figure 3-29 show the liquid chromatograms for the intantaneous ozonation of EE2 with isolated absorbance at 280 nm for both ESI negative and positive modes.

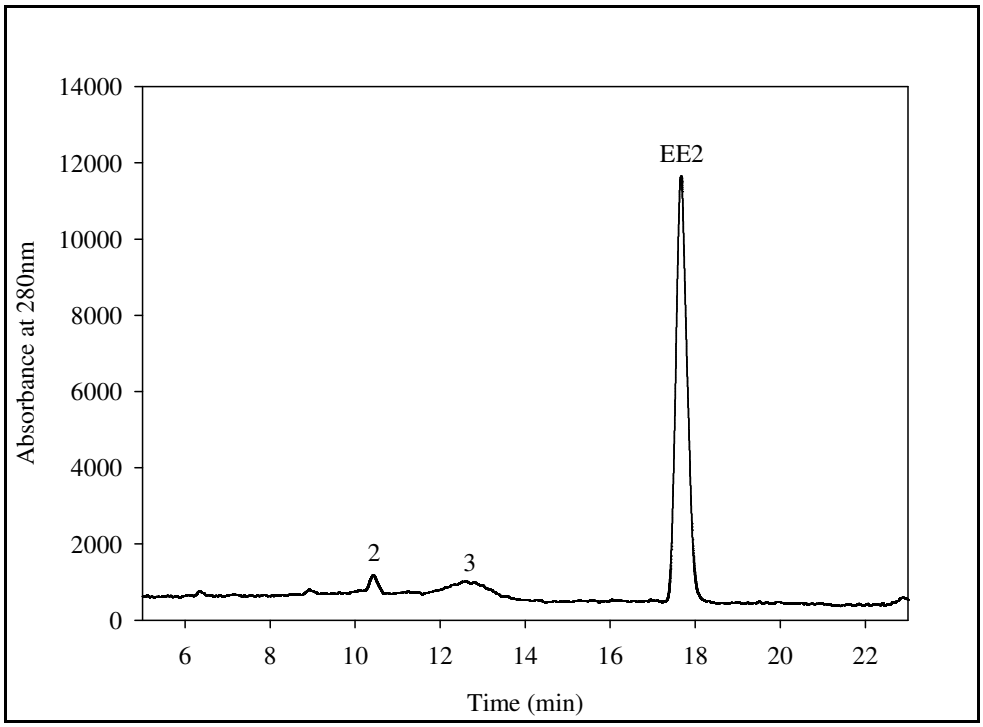


Figure 3-28: ESI Negative Isolated Absorbance at 280 nm Chromatogram of EE2 Instantaneous Ozonation

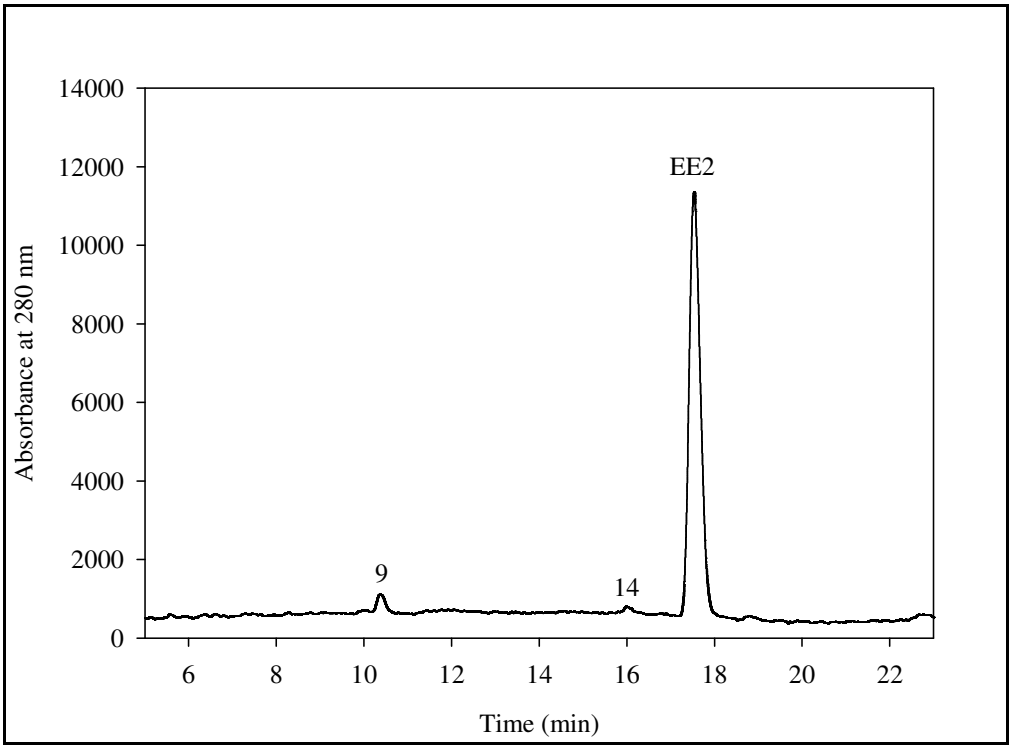


Figure 3-29: ESI Positive Isolated Absorbance at 280 nm Chromatogram of EE2 Instantaneous Ozonation

The byproducts formed from EE2 and short term O_3 reaction were observed at retention times of 6, 9, 10, 13, 15 and 19 min when the absorbance at 280 nm was isolated in the ESI negative mode. At an O_3 dose of 6 mg/L, 97 % of the EE2 concentration was degraded.

Figure 3-30 and Figure 3-31 show the liquid chromatograms for the short term ozonation of EE2 with isolated absorbance at 280 nm in the ESI negative and positive mode, respectively. From the isolated chromatograms, each peak was integrated to determine the associated areas.

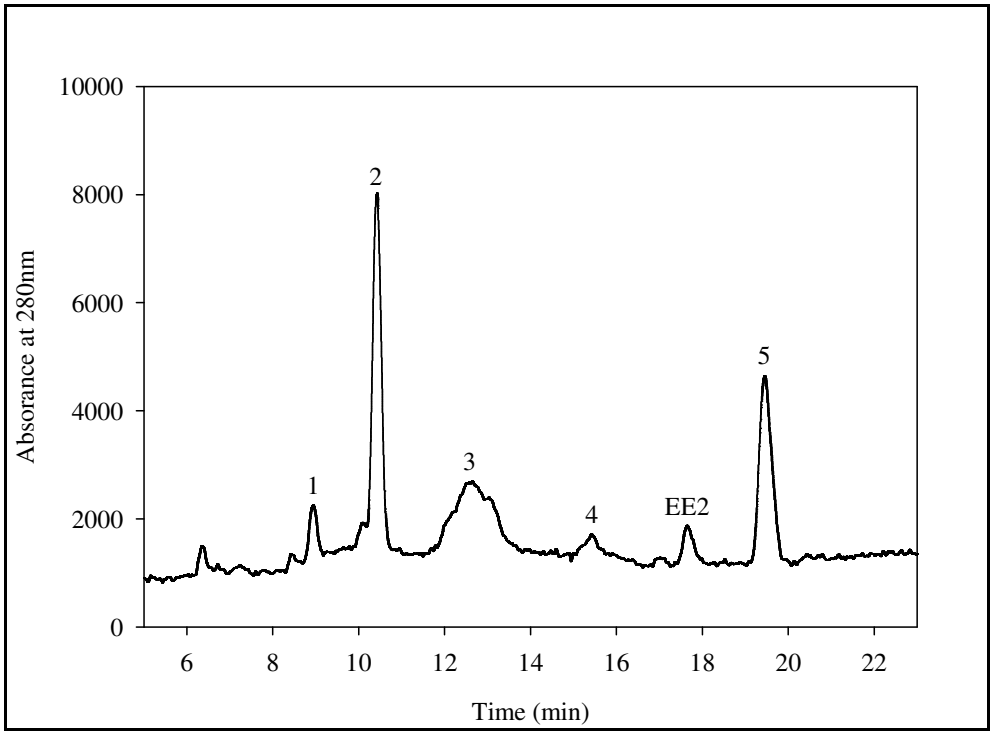


Figure 3-30: ESI Negative Chromatogram of EE2 Short Term Ozonation with Isolated Absorbance at 280 nm

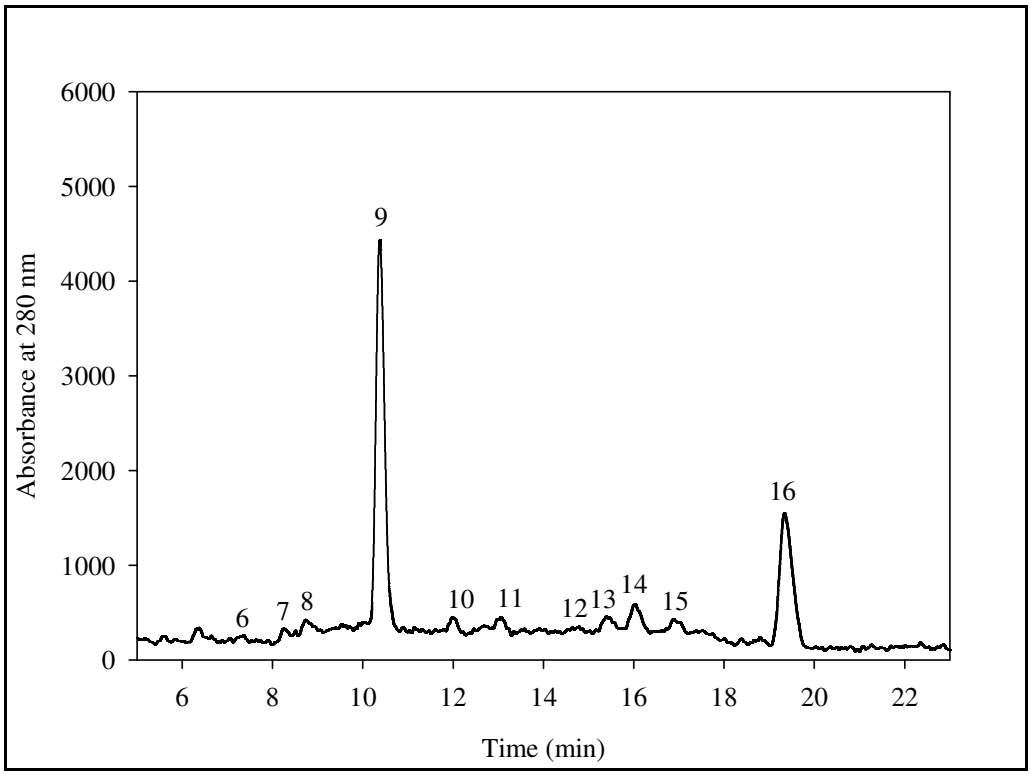


Figure 3-31: ESI Positive Chromatogram of EE2 Short Term Ozonation with Isolated Absorbance at 280 nm

The peak areas for the major byproducts and EE2 versus ozone dose are shown in Figure 3-32 and Figure 3-34 for the instantaneous ozonation reaction and for the short term ozonation reaction in ESI negative mode. Figure 3-33 and Figure 3-35 display the byproduct formation in ESI positive mode for the instantaneous and short term reaction, respectively.

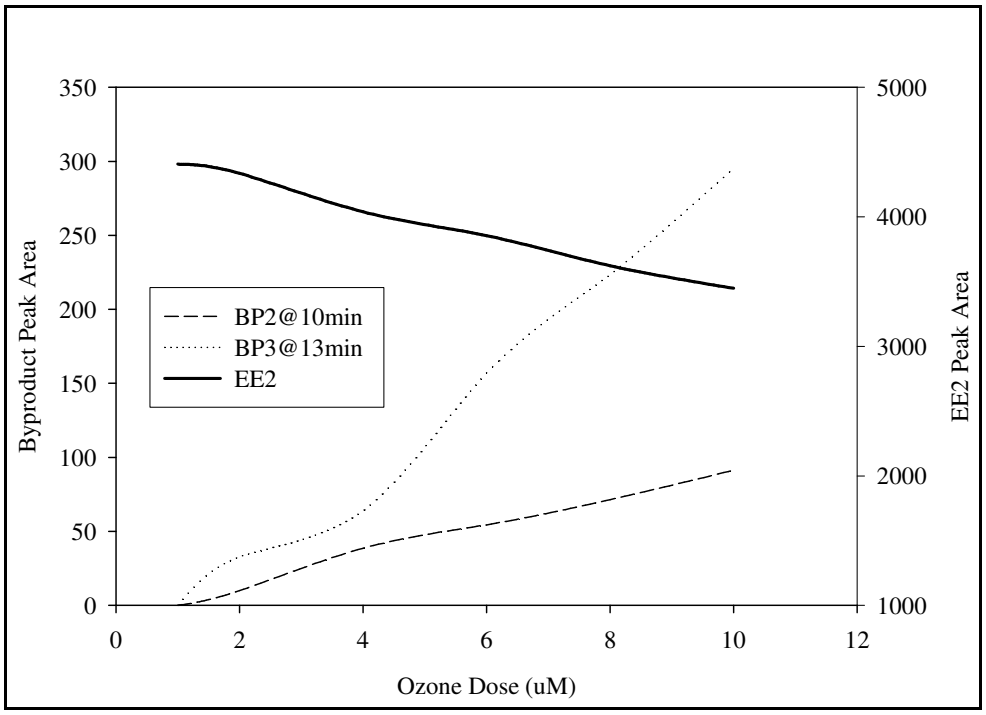


Figure 3-32: Formation of ESI Negative Major Byproducts from Instantaneous Ozonation of Ethinylestradiol

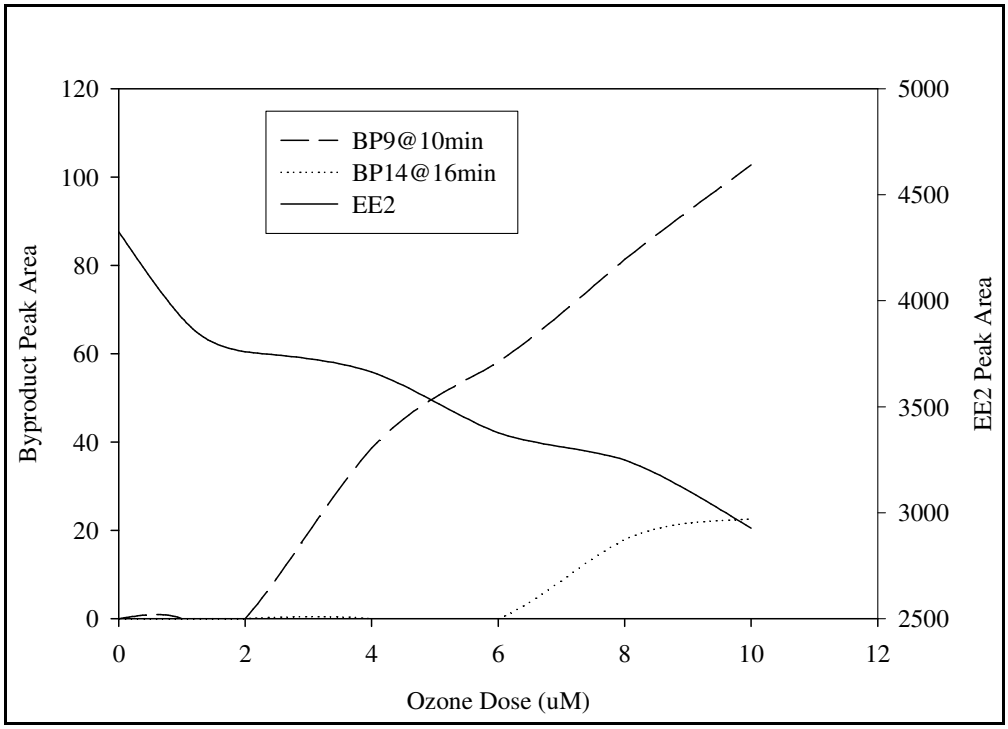


Figure 3-33: Formation of ESI Positive Major Byproducts from Instantaneous Ozonation of Ethinylestradiol

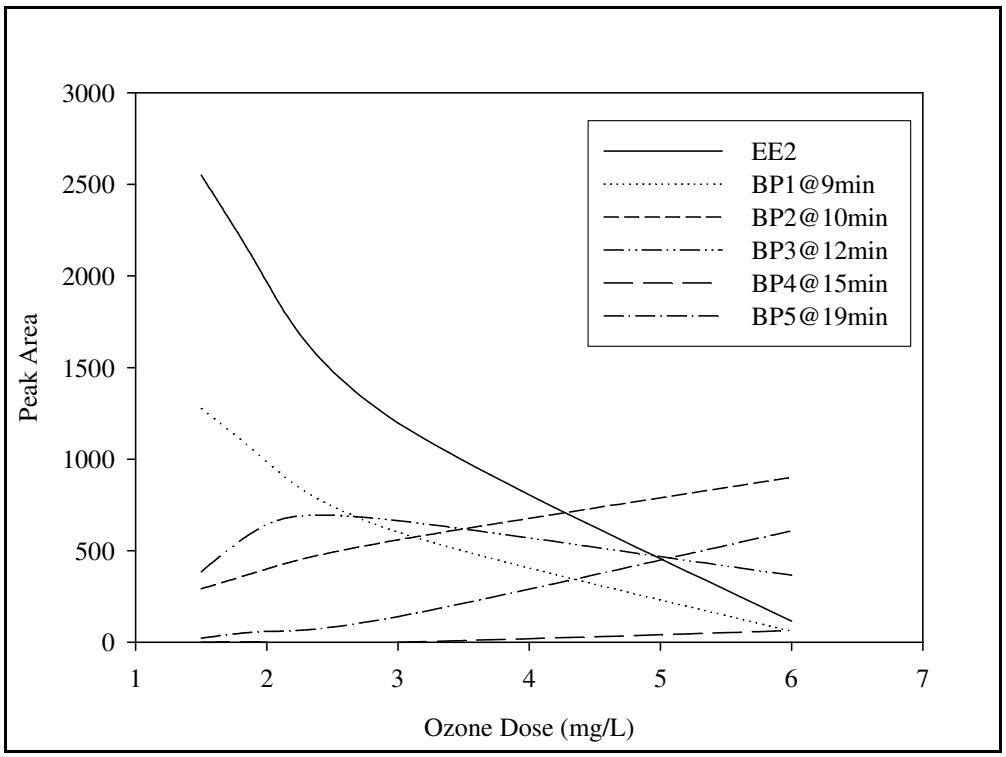


Figure 3-34: Formation of ESI Negative Major Byproducts from Short Term Ozonation of Ethinylestradiol

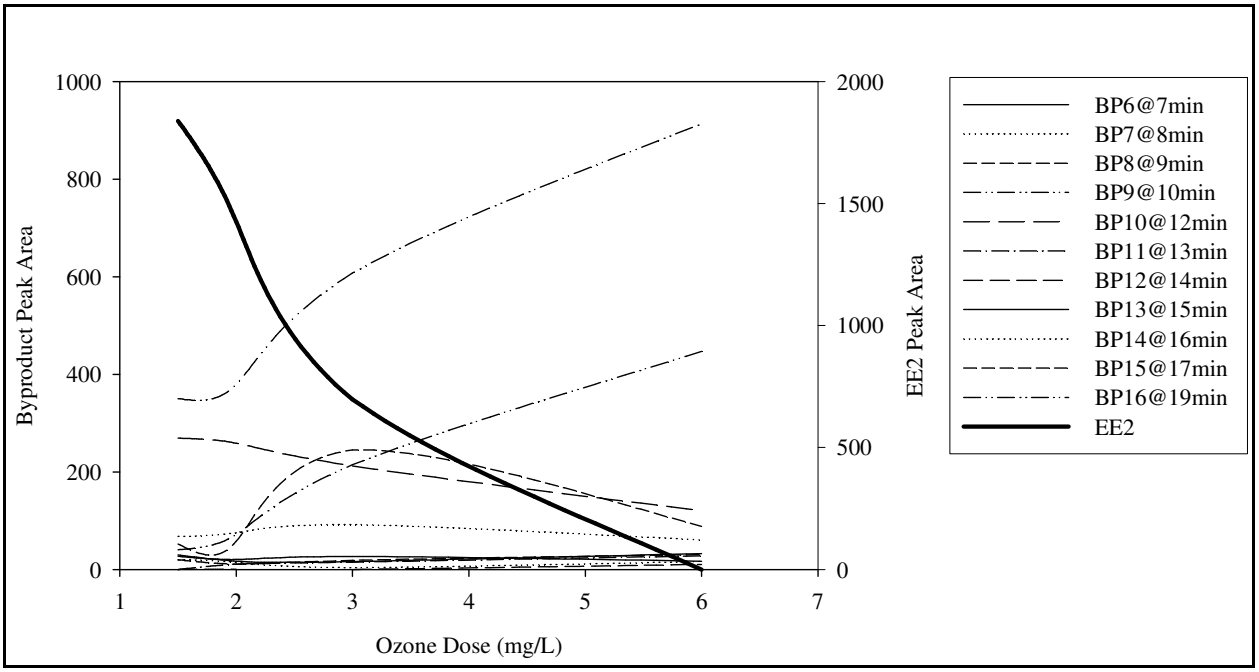


Figure 3-35: Formation of ESI Positive Major Byproducts from Short Term Ozonation of Ethinylestradiol

As the ozone dose increased, EE2 followed a linear decay function. The byproduct at 9 min was formed immediately after the lowest ozone dose was administered, then started to decrease after the ozone dose was increased. Byproducts found at 10 and 19 min followed a linear growth function. The byproduct at 12 minutes increased in quantity until the 3.0 mg/L dose was administered, then a drop in byproduct at 12 min was observed. The byproduct at 15 min formed at a slower rate and a lower yield as compared to the other byproducts.

Identification

The byproducts at 15 and 19 min contain two local maximum in the corresponding absorbance spectrum, following the EE2 pattern. The byproducts found at 9, 10 and 13 min show a single local maximum pattern. Byproducts at 9, 10, 13 and 15 min eluted prior to EE2 when using a mobile phase of methanol and water and BP at 19 min eluted after EE2. The absorbance spectra for EE2 and the observed ozonation byproducts are presented in Figure 3-36, Figure 3-37 and Figure 3-38.

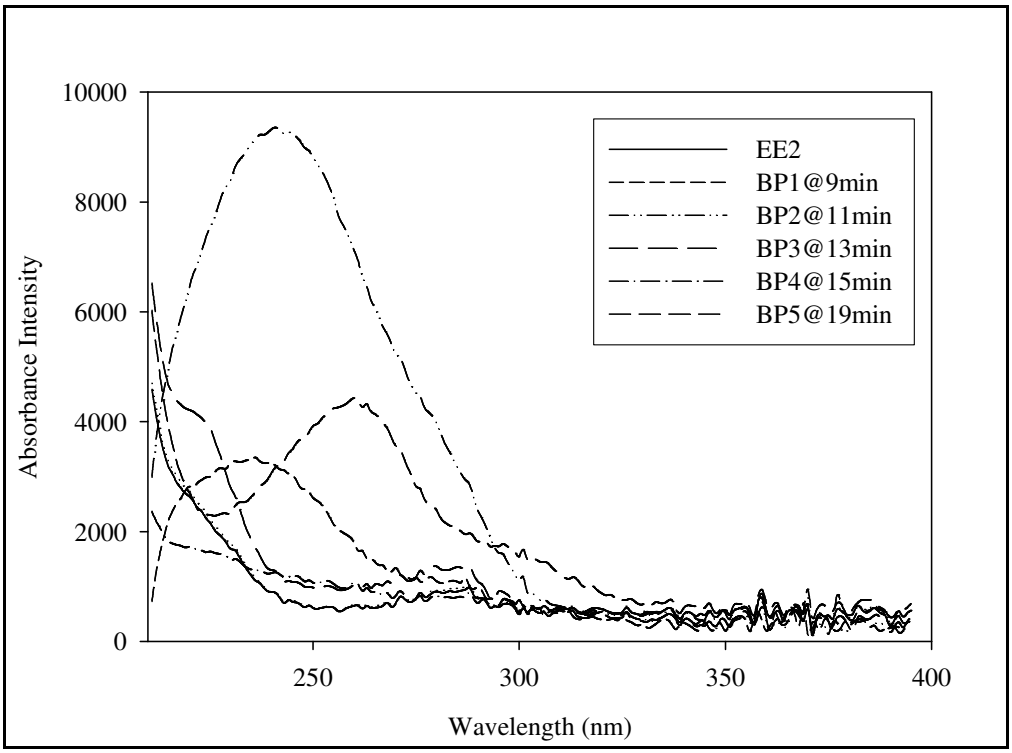


Figure 3-36: Absorbance of EE2 and ESI Negative Ozonation Byproducts

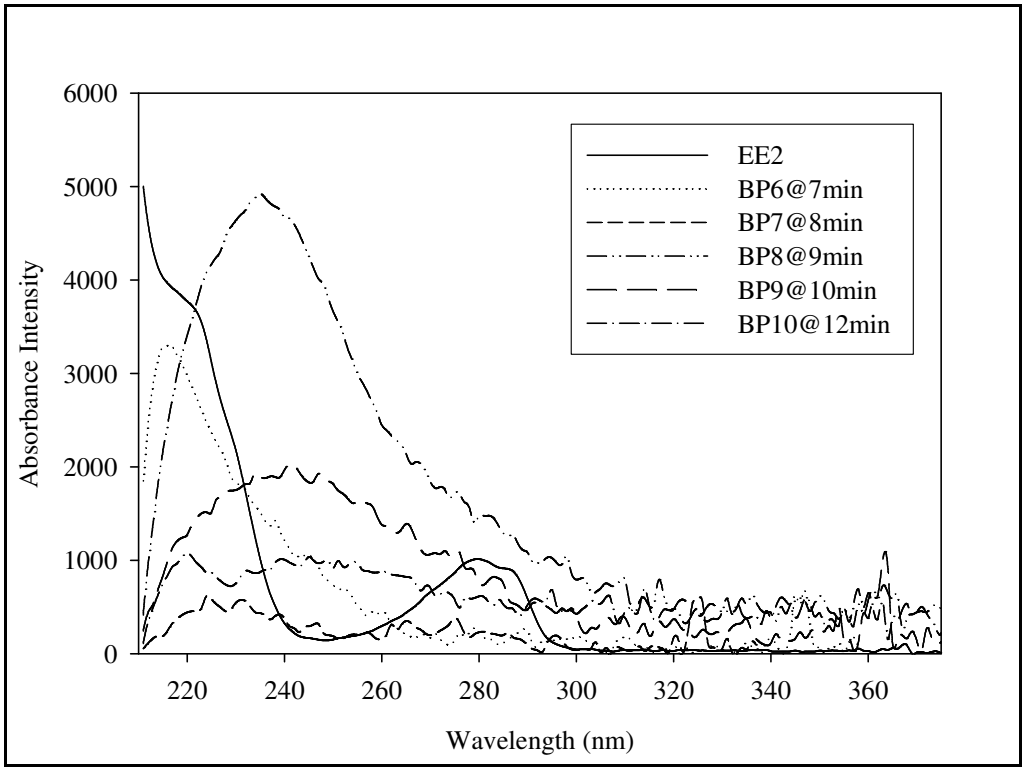


Figure 3-37: Absorbance of EE2 and ESI Positive Ozonation Byproducts 6 through 10

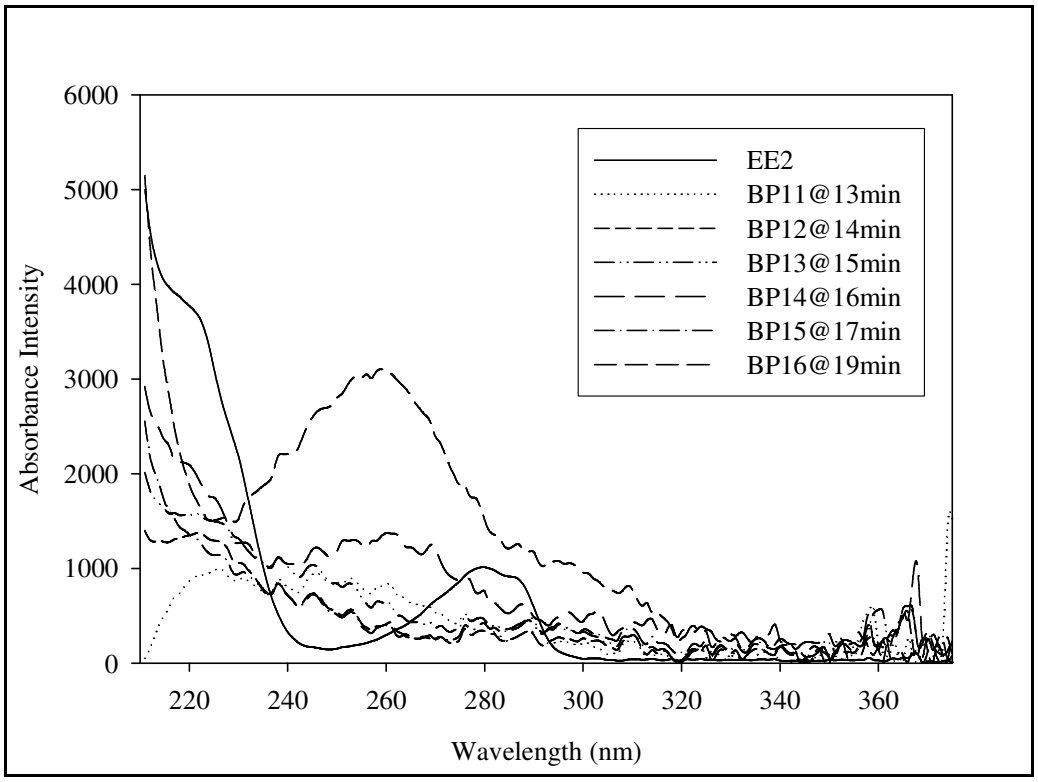


Figure 3-38: Absorbance of EE2 and ESI Positive Ozonation Byproducts 11 through 16

Based on the retention times and corresponding polarity, mass spectra and absorbance at 280 nm, the major byproducts were identified for the ozonation of EE2 and presented in Table 3-8 and Table 3-9.

Table 3-8: Proposed Identification of EE2 and ESI Negative Ozonation Byproducts

Analyte	Retention Time (min)	Identity
EE2	17 min	Ethinylestradiol
Byproduct 1	9 min	$C_{20}H_{24}O_3$ MW 312 m/z
Byproduct 2	10 min	$C_{20}H_{24}O_3$ MW 312 m/z
Byproduct 3	13 min	$C_{20}H_{22}O_4$ MW 326 m/z
Byproduct 4	15 min	MW 339 m/z
Byproduct 5	19 min	$C_{20}H_{22}O_3Cl_2$ MW 379 m/z

Table 3-9: Proposed Identification of EE2 and ESI Positive Ozonation Byproducts

Analyte	Retention Time (min)	Identity
EE2	17 min	Ethinylestradiol
Byproduct 6	7 min	$C_{20}H_{22}O_6$ MW 358 m/z
Byproduct 7	8 min	$C_{20}H_{27}O_8$ MW 398 m/z
Byproduct 8	9 min	$C_{20}H_{22}O_5$ MW 342 m/z
Byproduct 9	10 min	$C_{20}H_{16}O_7$ MW 368 m/z
Byproduct 10	12 min	$C_{20}H_{24}O_4$ MW 328 m/z
Byproduct 11	13 min	$C_{20}H_{18}O_7$ MW 370 m/z
Byproduct 12	14 min	$C_{20}H_{26}O_6$ MW 362 m/z
Byproduct 13	15 min	$C_{20}H_{23}O_2Cl_3$ MW 400 m/z
Byproduct 14	16 min	MW 353 m/z
Byproduct 15	16.8 min	MW 353 m/z
Byproduct 16	19 min	$C_{20}H_{22}O_2Cl_4$ MW 435 m/z

Sixteen byproducts were formed during the ozonation of EE2; five of these byproducts were detected using ESI negative and eleven of the byproducts were detected using ESI positive. These byproduct formations were the result of oxygen radicals or molecular ozone attacking the phenolic moiety at the ortho position(s), with respect to the hydroxyl group at C3. Oxygen radicals may also have attacked the five carbon ring and breaking the bond between C16 and C17, creating a carboxylic acid group on C17. Based on the isotope pattern for byproducts 5, 13 and 16, chlorinated ozonation byproducts were produced as a result of dilute levels of HCl (10^{-4} M) in the ozone stock solution, which aided in the increase of the ozone stock concentration. Byproducts 4, 14 and 15 were not identified due to the insufficient fragmentation information on the mass spectra.

The mass spectra for EE2 and each ozonation byproduct formed are located in Appendix B. The proposed reactions between ozone and EE2 are shown in Figure 3-39 and Figure 3-40, where solid arrows indicate identified byproducts and dashed arrows signify potential byproducts.

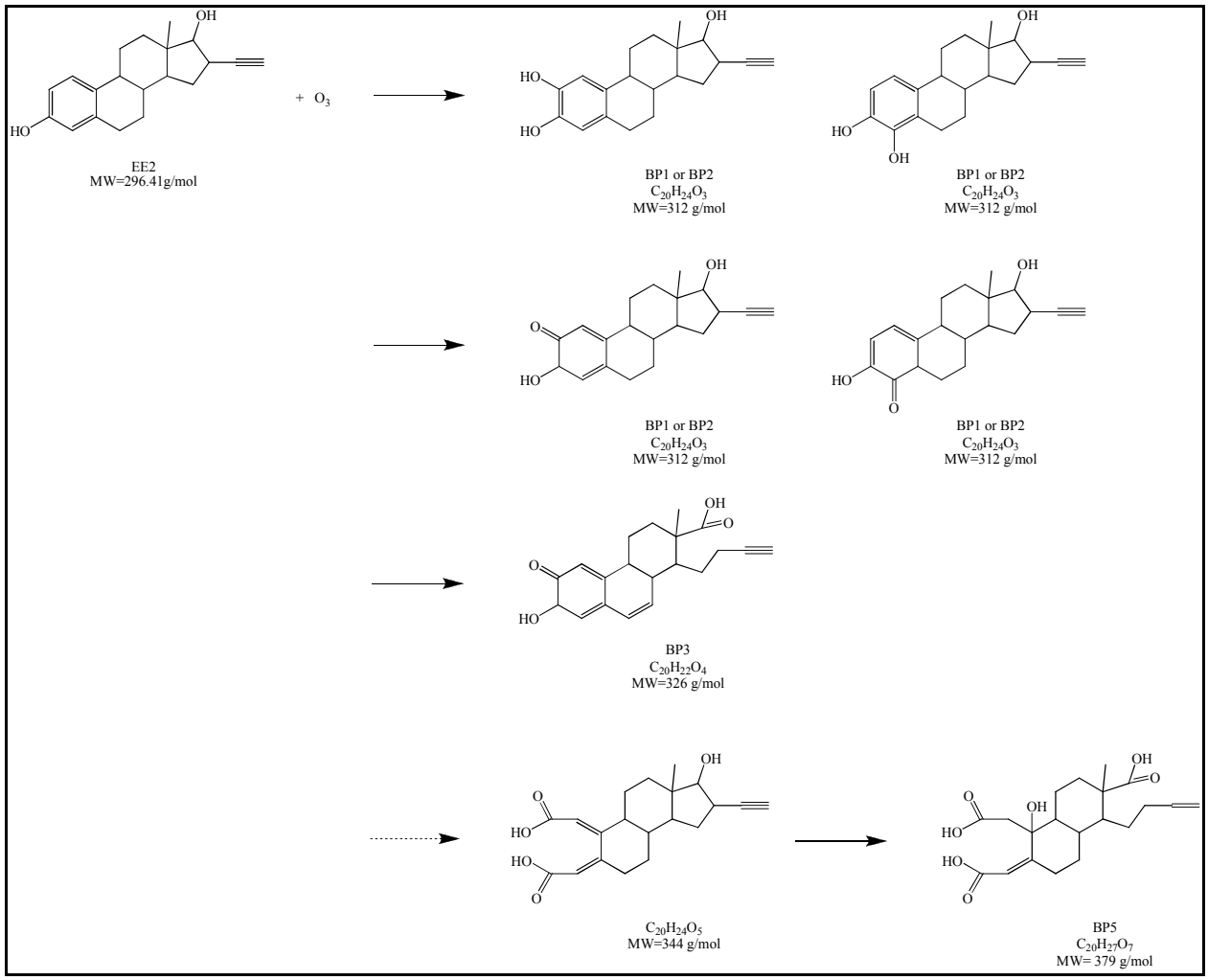


Figure 3-39: Proposed ESI Negative Byproducts from the O_3 and EE2 Reaction

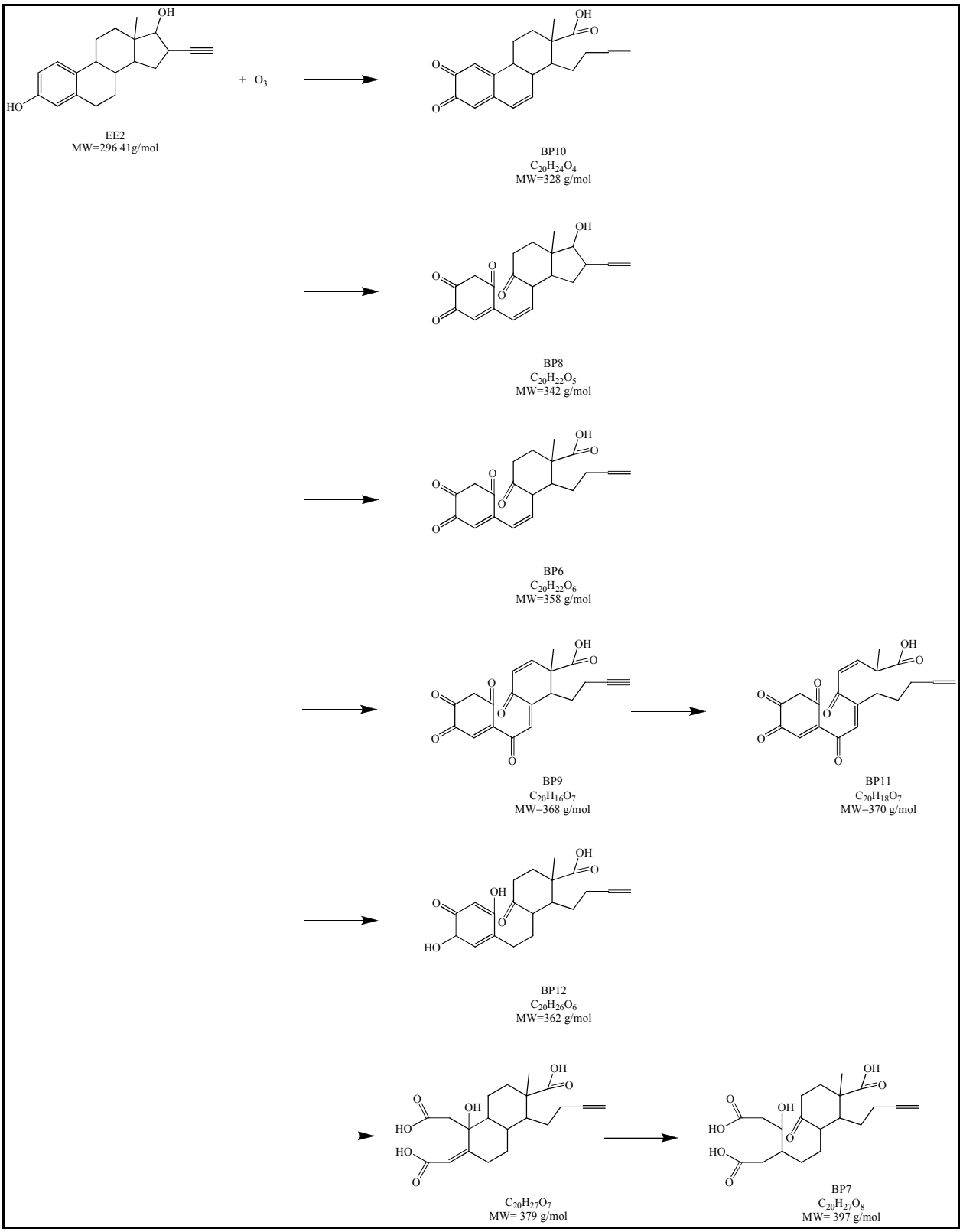


Figure 3-40: Proposed ESI Positive Byproducts from the O_3 and EE2 Reaction

The byproducts found in ESI negative are similar to those suggested by Lee *et al* (2008) and Zhang *et al.* (2006); however many more possible byproducts were determined due to second reaction step or ozonation of preliminary byproducts that were detected.

The chlorinated ozonation byproducts are shown in Figure 3-41.

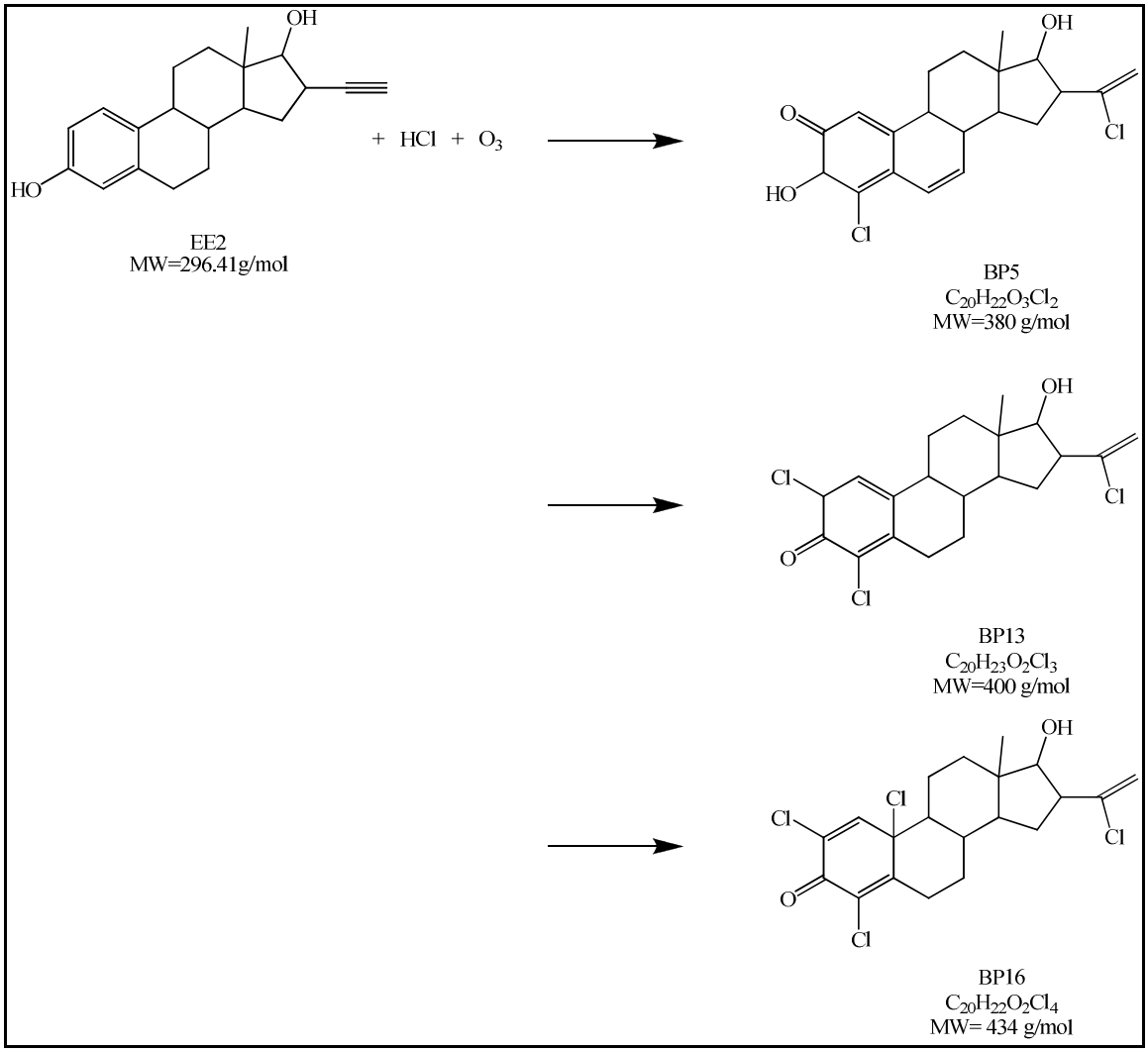


Figure 3-41: Proposed Chlorinated Ozonation Byproducts from the EE2 Reaction

Relative Quantification

Since the ozone stock solution did not reach a highly concentrated solution, even when using an ice bath, the short term ozonation doses were relatively high compared to the other treatment doses. This larger dose volume resulted in a dilution of the sample solutions and an artificial decrease in EE2 concentration for the short term reactions.

The relative concentration of each ozonation byproduct was determined based on peak areas since authentic standards were unavailable for each byproduct. For purposes of relative concentrations, it was assumed that the only byproducts formed during the reactions were those that absorbed at a wavelength of 280 nm. The estimated quantification of each byproduct was based on the initial concentration of EE2 (10.0 ± 1.0 μM) and the molecular weights found for each byproduct. Figure 3-42 (ESI negative) and Figure 3-43 (ESI Positive) provide EE2 and its ozonation byproducts with respect to dose.

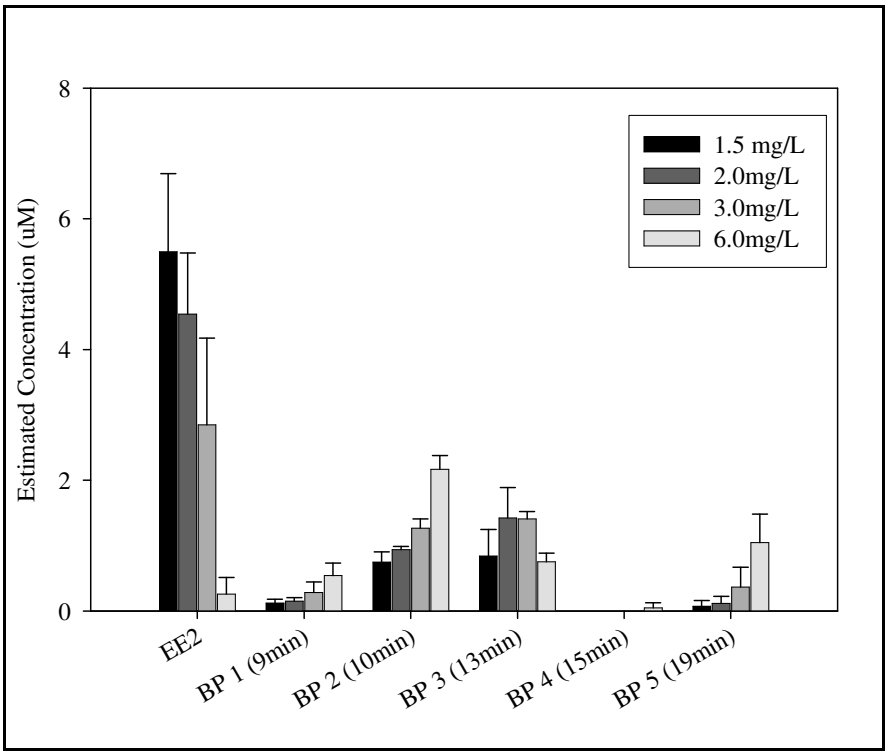


Figure 3-42: Estimated Concentrations of EE2 Short Term Ozonation Byproducts (ESI Negative)

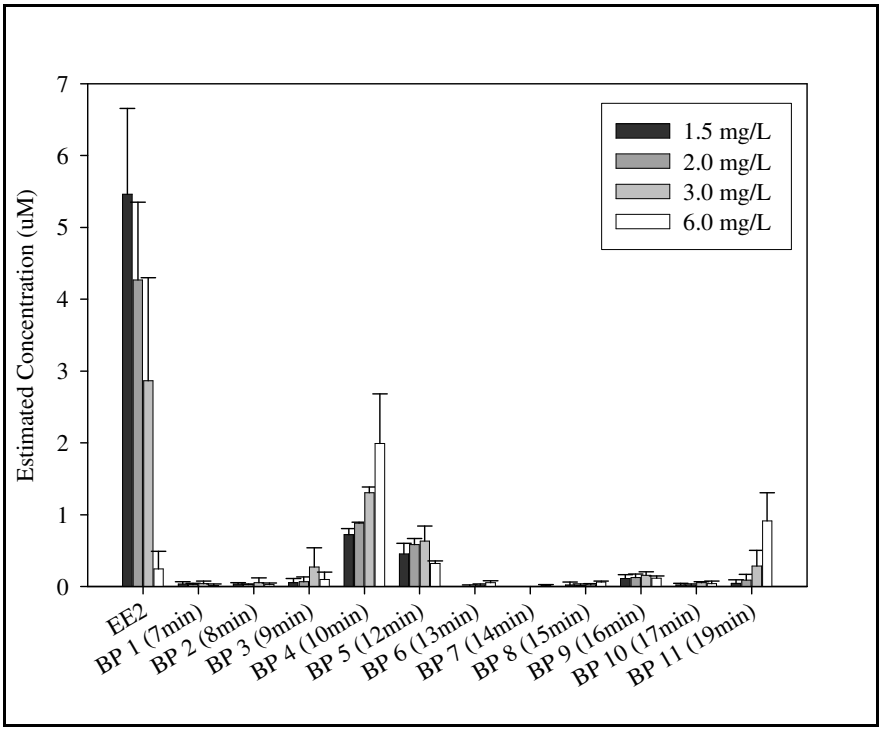


Figure 3-43: Estimated Concentrations of EE2 Short Term Ozonation Byproducts (ESI Positive)

The instantaneous ozonation reactions showed an EE2 degradation of 23 % for 0.5 mg/L O_3 instantaneous dose. For the short-term reactions with a dose of 6.0 mg/L O_3, EE2 provided a degradation of 97 % according to the ESI negative peak areas.

3.5.3.2 Ozonation of DES

Reactions

Figure 3-44 and Figure 3-50 show DES and total byproducts peak areas versus ozone dose, which resulted from the instantaneous and short term ozonation of DES in ESI negative, respectively. Figure 3-45 and Figure 3-47 provide the peak areas for the ESI positive byproducts of DES ozonation. Each experiment included initial concentrations of DES of 10.0 ± 1.0 µM.

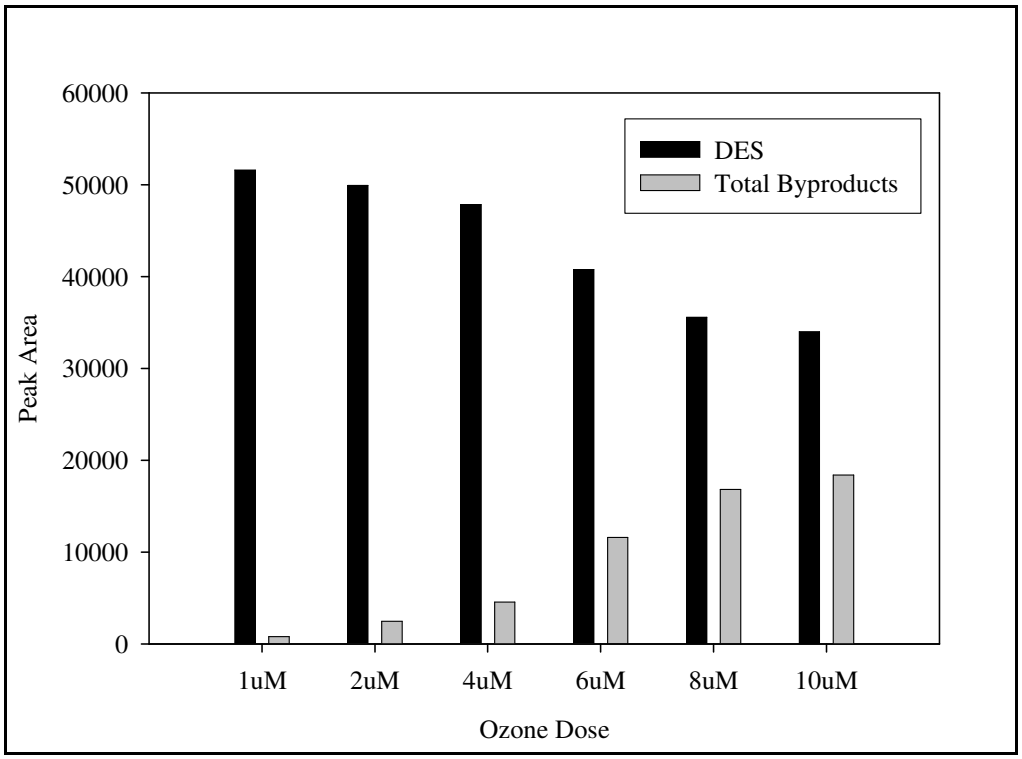


Figure 3-44: Instantaneous Ozonation of DES (ESI Negative)

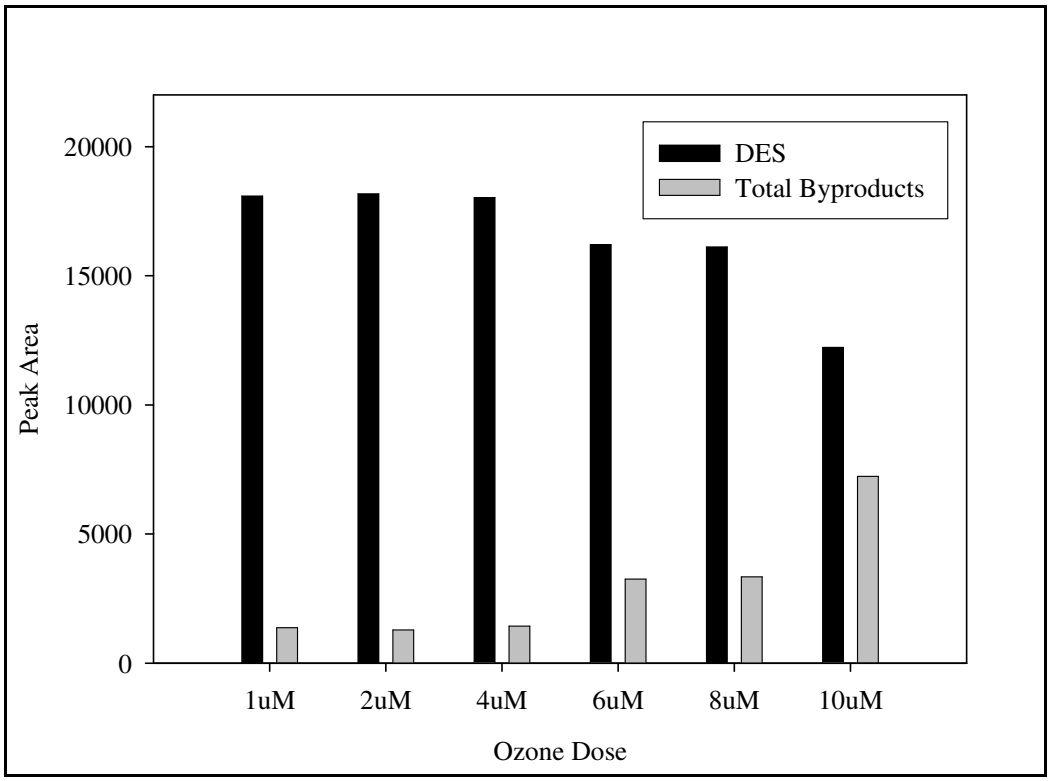


Figure 3-45: Instantaneous Ozonation of DES (ESI Positive)

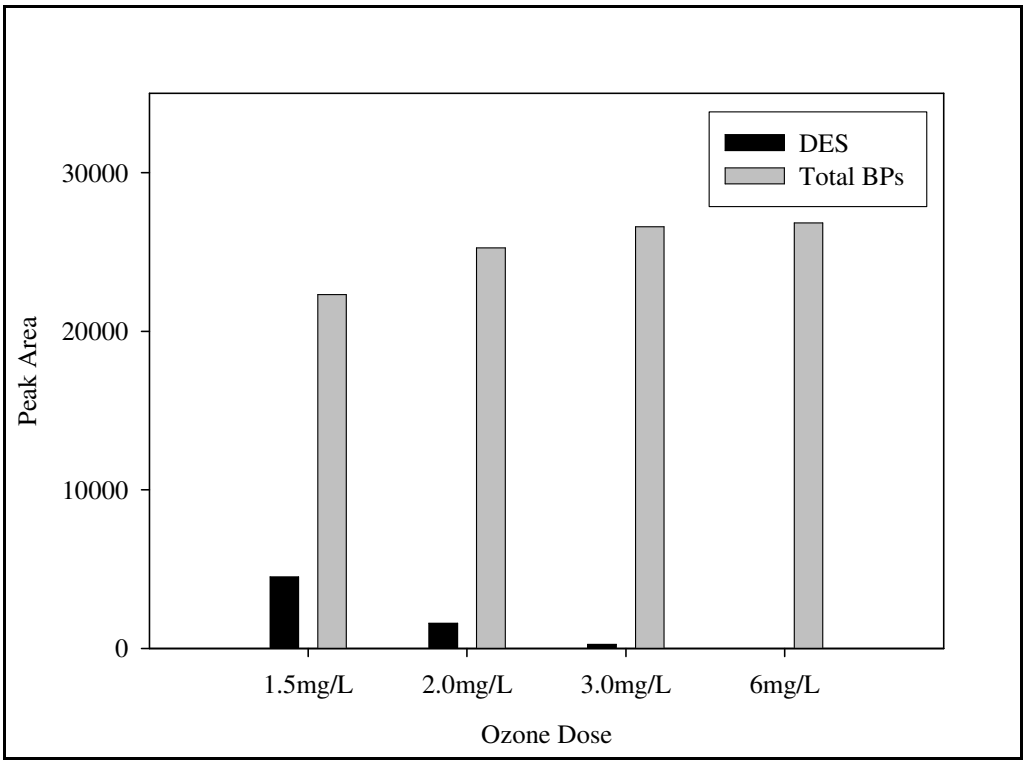


Figure 3-46: Short Term Ozonation of DES (ESI Negative)

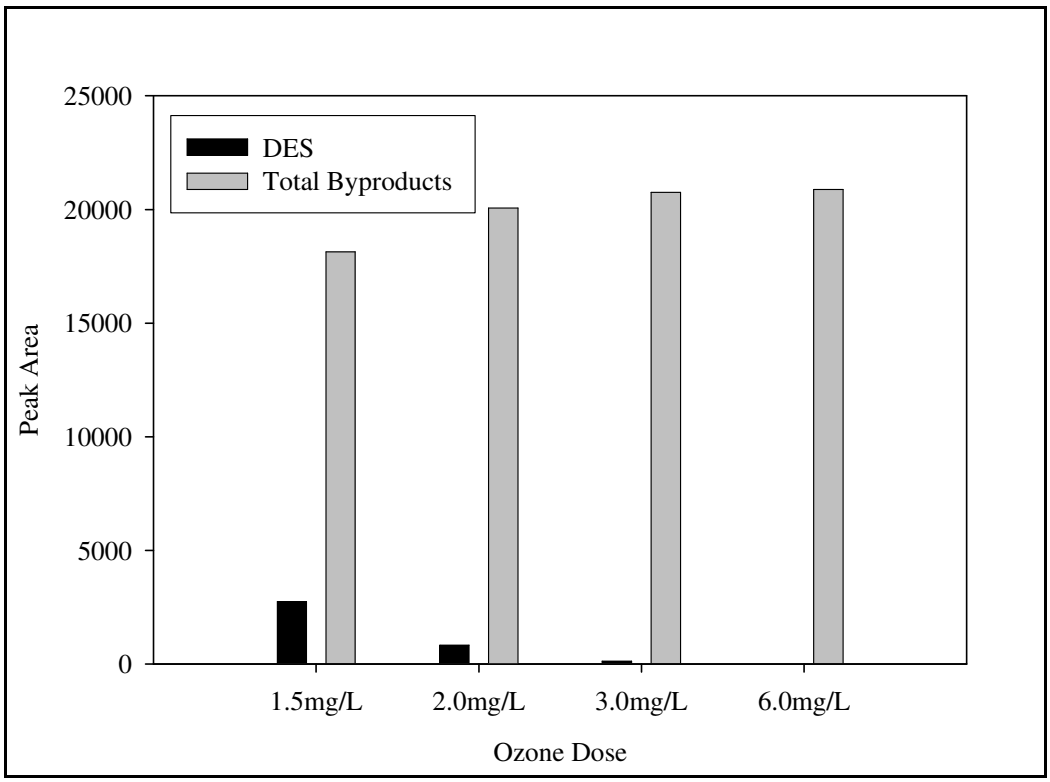


Figure 3-47: Short Term Ozonation of DES (ESI Positive)

For each ozonation reaction, it was observed that as DES was degraded, the total peak area of the byproducts increased. As the ozone dose was increased, the DES concentration also decreased in similar proportions to the O_3 added for the disinfection reaction. Results for each byproduct separated for individual ozone dose is presented in Appendix B for both the instantaneous and short term ozonation of DES.

Detection

DES followed a curvalinear decay function for both the instantaneous and short term ozonation reactions. The byproducts formed from DES and the instantaneous O_3 reactions were observed at 8, 9, 11, 12, 13, 14, 15 and 19 min on the chromatogram when the absorbance at 240 nm was

isolated. At an O_3 dose of 10 μM, 35 % of the DES concentration was degraded. At an O_3 dose of 6 mg/L, 100 % of the DES degraded.

Figure 3-48 and Figure 3-49 show the liquid chromatograms for the intantaneous ozonation of DES with isolated absorbance at 240 nm in ESI negative and positive, respectively.

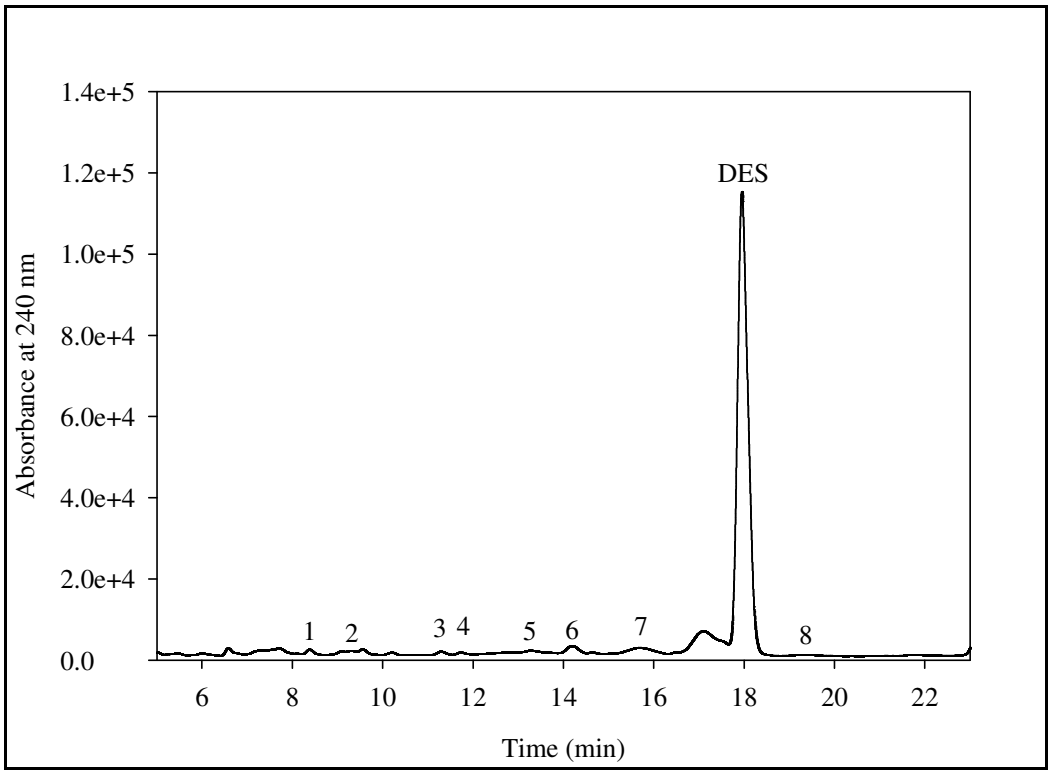


Figure 3-48: Isolated Absorbance at 240 nm Chromatogram of DES Instantaneous Ozonation (ESI Negative)

The byproducts formed from DES and instantaneous O_3 reaction were observed at 7, 8, 9, 10, 12, 13, 14, 15, 17, 19 and 21 min on the ESI positive chromatogram when the absorbance at 240 nm was isolated.

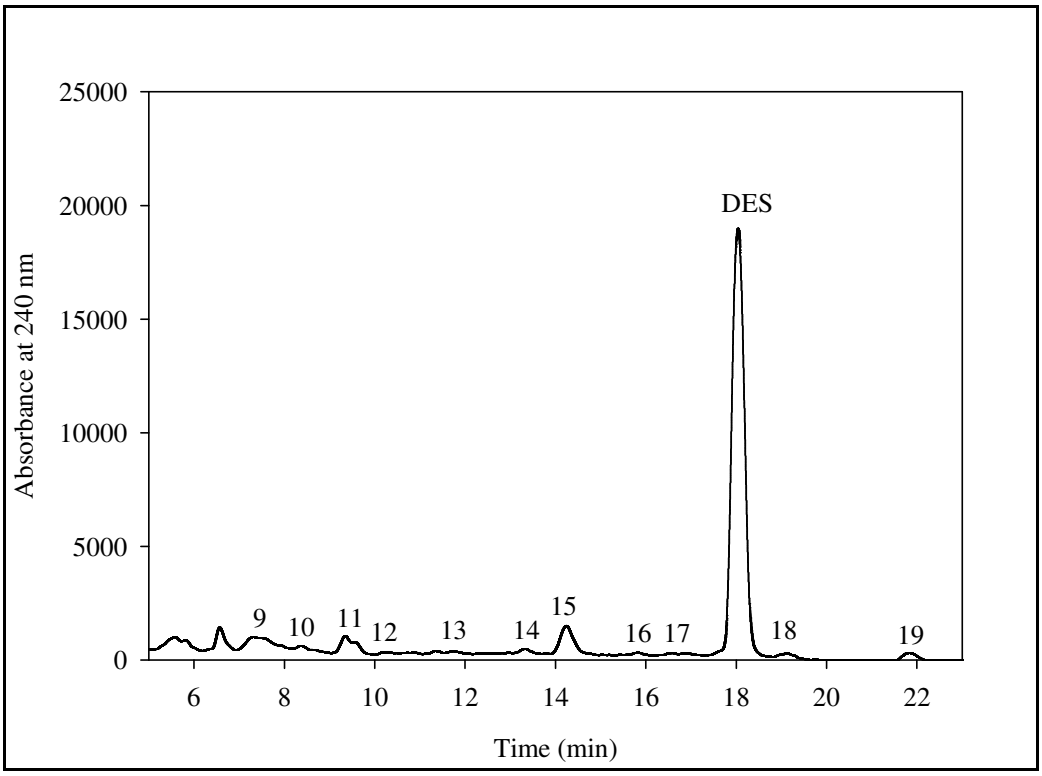


Figure 3-49: Isolated Absorbance at 240 nm Chromatogram of DES Instantaneous Ozonation (ESI Positive)

The byproducts formed from DES and short term O_3 reaction were observed at 8, 9, 11, 12, 13, 14, 16 and 19 min on the ESI negative chromatogram when the absorbance at 240 nm was isolated. With an O_3 dose of 6.0 mg/L, DES was not detected by LC/MS; therefore, it was determined that 100 % of the concentration was degraded. The byproducts formed from DES and short term O_3 reaction were observed at 7, 8, 9, 10, 12, 13, 14, 15, 17, 19 and 21 min on the ESI positive chromatogram when the absorbance at 240 nm was isolated.

Figure 3-50 and Figure 3-51 show the liquid chromatograms for the short term ozonation of DES with isolated absorbance at 240 nm in ESI negative and positive, respectively. From the isolated chromatograms, each peak was integrated to determine the associated areas.

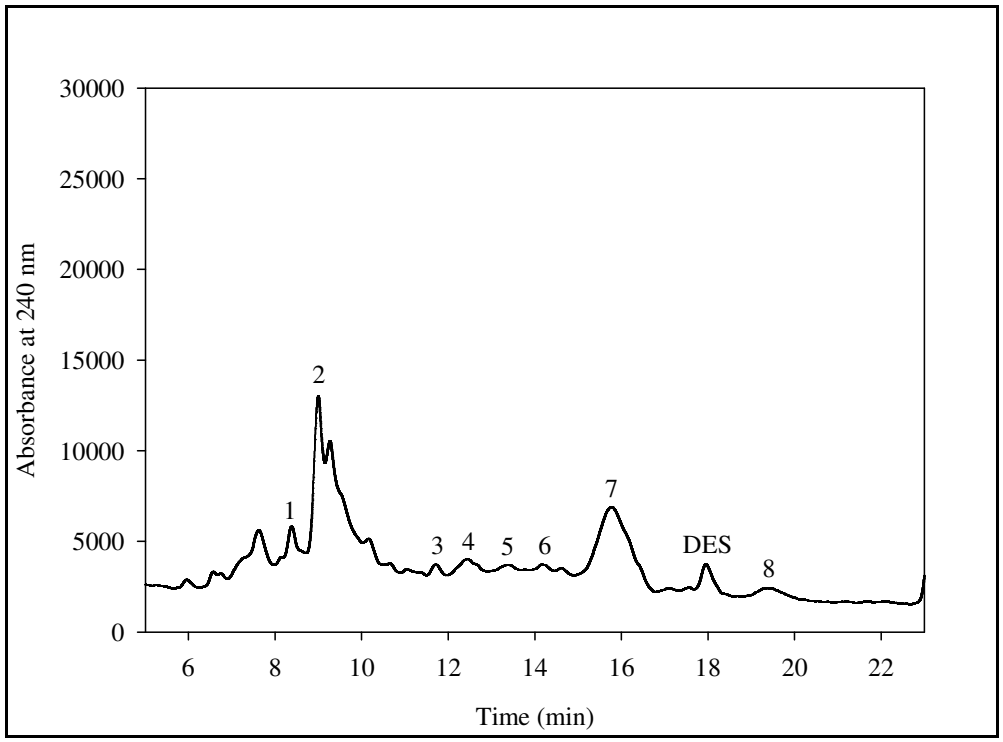


Figure 3-50: Chromatogram of DES Short Term Ozonation with Isolated Absorbance at 240 nm (ESI Negative)

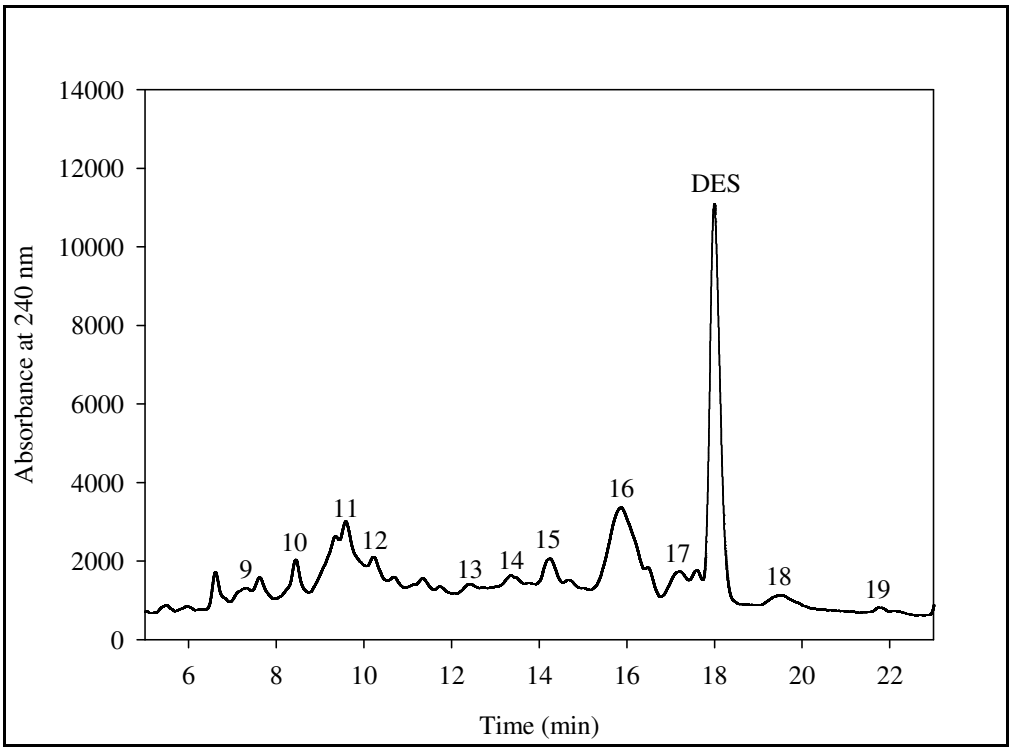


Figure 3-51: Chromatogram of DES Short Term Ozonation with Isolated Absorbance at 240 nm (ESI Positive)

The peak areas for the major byproducts and DES versus ozone dose are shown in for the Figure 3-52, Figure 3-53, Figure 3-54 and Figure 3-55 instantaneous ozonation reaction and for the short term ozonation reaction.

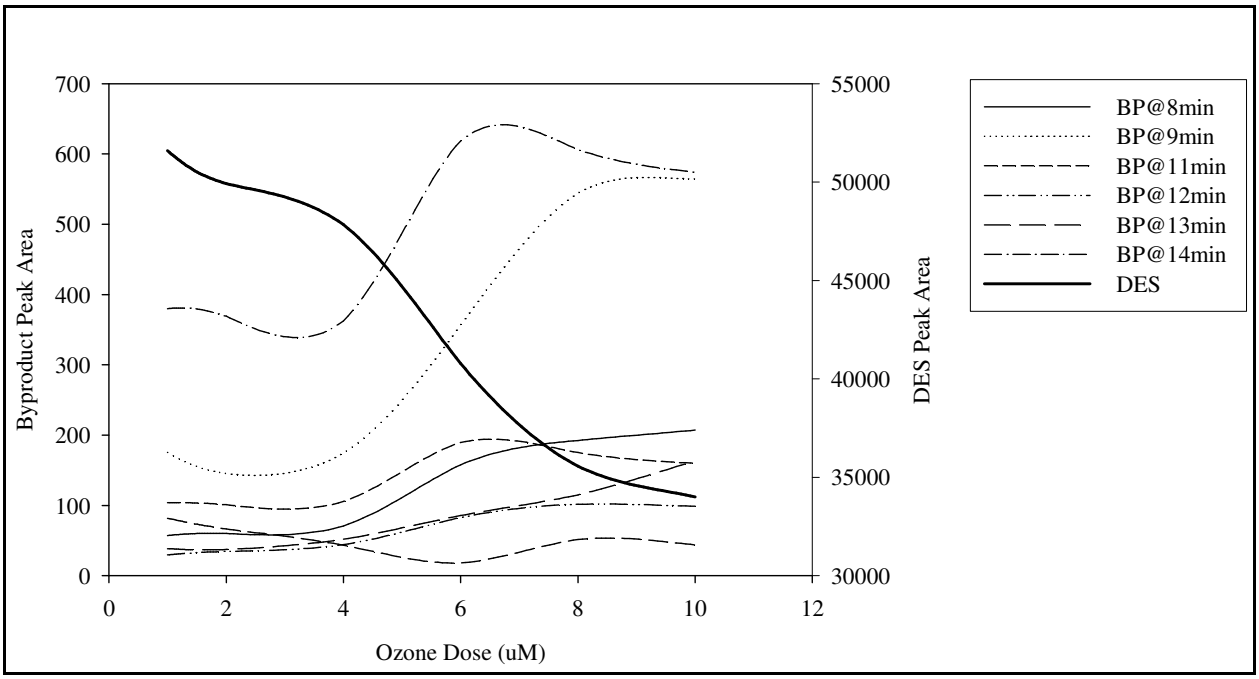


Figure 3-52: Formation of Major Byproducts from Instantaneous Ozonation of Diethylstilbestrol (ESI Negative)

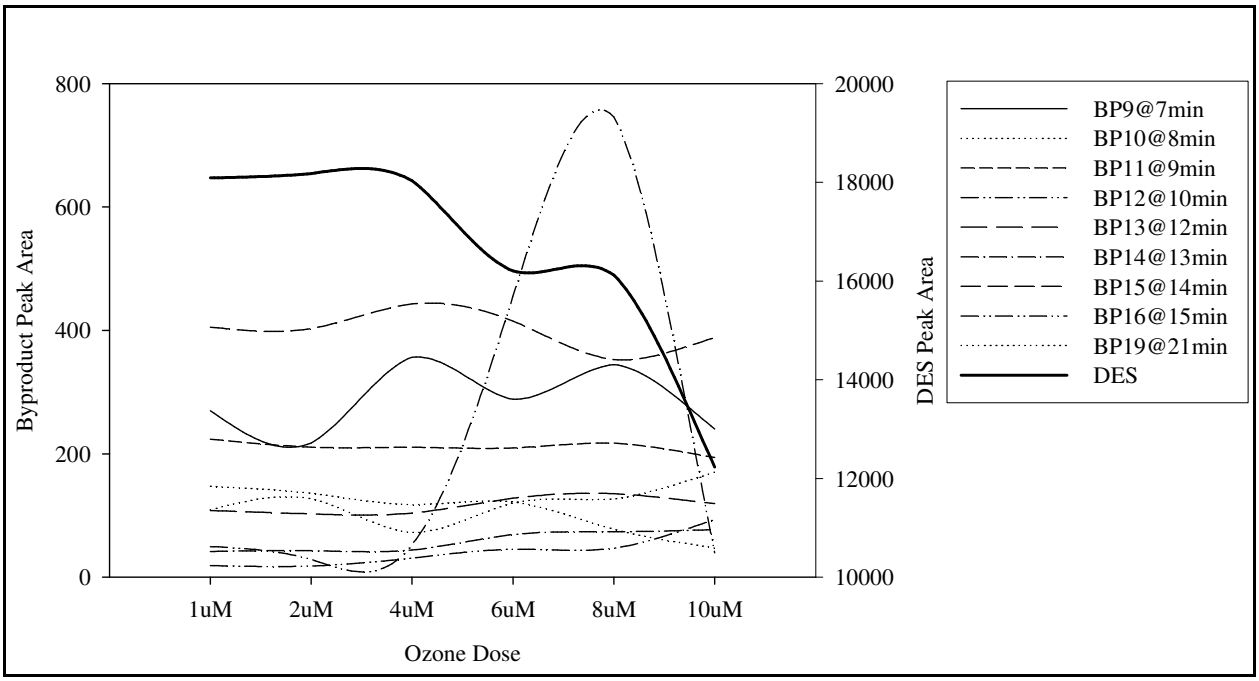


Figure 3-53: Formation of Major Byproducts from Instantaneous Ozonation of Diethylstilbestrol (ESI Positive)

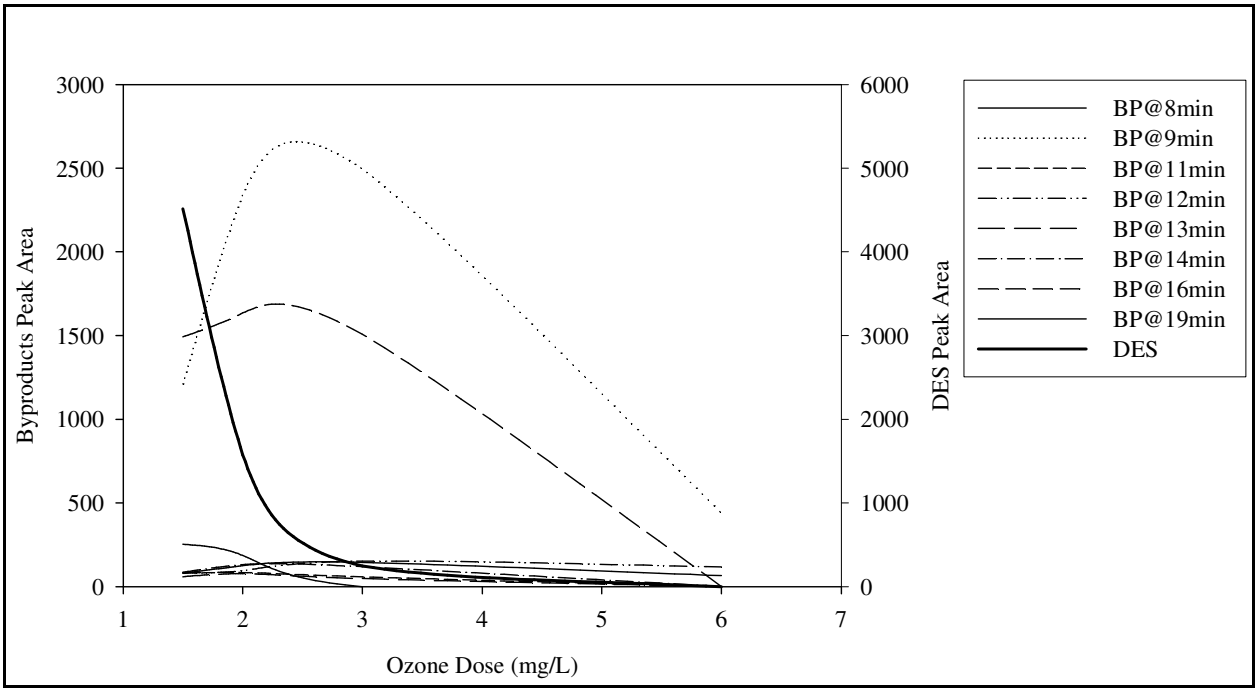


Figure 3-54: Formation of Major Byproducts from Short Term Ozonation of Diethylstilbestrol (ESI Negative)

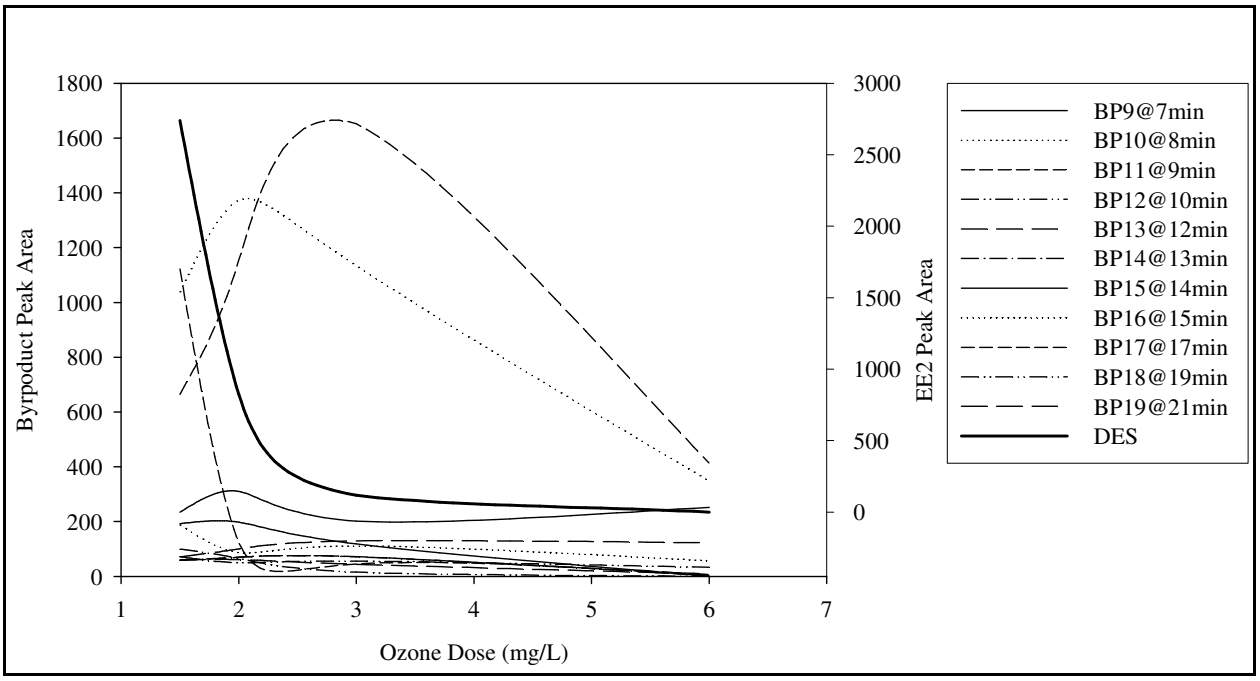


Figure 3-55: Formation of Major Byproducts from Short Term Ozonation of Diethylstilbestrol (ESI Positive)

Identification

The byproducts found at 11, 12, 13, 14, 16, 19 and 22 min present the same local maximum pattern as DES. The byproducts at 8 and 9 min also contain one local maximum in the corresponding absorbance spectrum; however, the maxima are located over 310 nm. BPs at 8, 9, 11, 13, 14 and 16 min eluted prior to DES when using a mobile phase of methanol and water and BPs at 19 and 22 min eluted after DES. The absorbance spectra for DES and the observed ozonation byproducts are shown in Figure 3-56 and Figure 3-57.

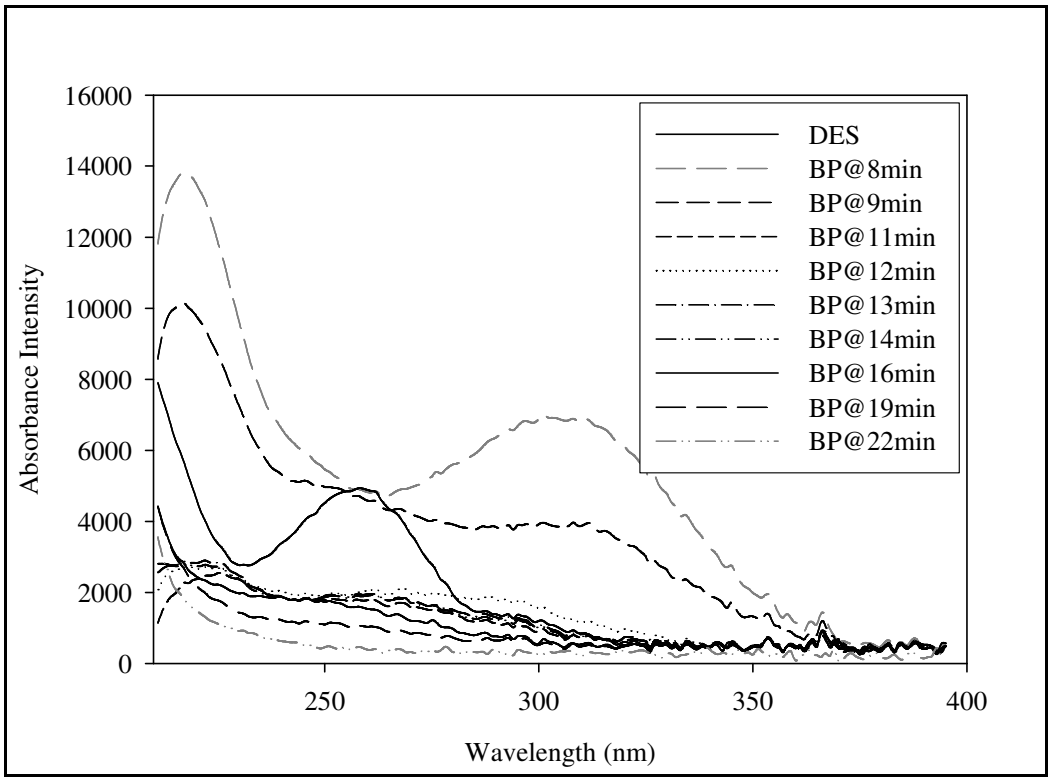


Figure 3-56: Absorbance of DES and Ozonation Byproducts in ESI Negative Mode

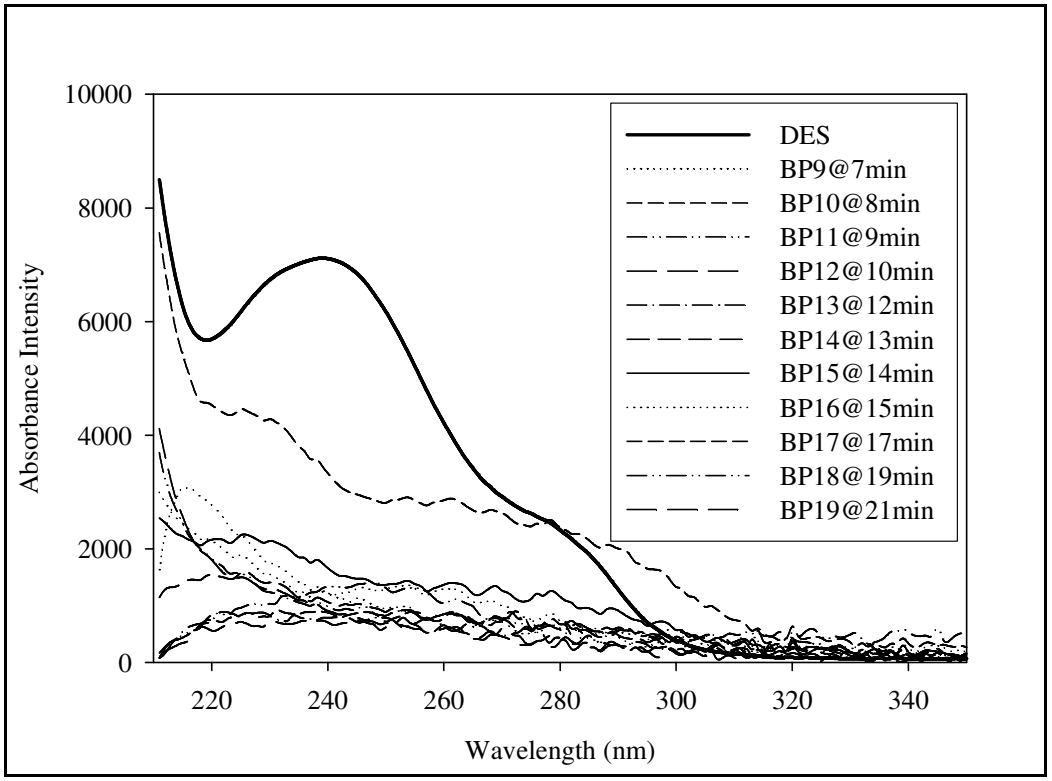


Figure 3-57: Absorbance of DES and Ozonation Byproducts in ESI Positive Mode

Based on the retention times and corresponding polarity, mass spectra and absorbance at 240 nm, the major byproducts were identified for the ozonation of DES and presented in Table 3-10 and Table 3-11.

Table 3-10: Proposed Identification of DES and ESI Negative Ozonation Byproducts

Analyte	Retention Time (min)	Identity
DES	18 min	Diethylstilbestrol
Byproduct 1	8 min	$C_{18}H_{20}O_3$ MW 284 m/z
Byproduct 2	9 min	$C_{18}H_{19}O_4Cl$ MW 335 m/z
Byproduct 3	11 min	$C_{18}H_{20}O_3$ MW 284 m/z
Byproduct 4	12 min	$C_{18}H_{20}O_3$ MW 284 m/z
Byproduct 5	13 min	$C_{18}H_{20}O_3$ MW 284 m/z
Byproduct 6	14 min	$C_{18}H_{20}O_3$ MW 284 m/z
Byproduct 7	16 min	$C_{18}H_{20}O_4$ MW 299 m/z
Byproduct 8	19 min	$C_{18}H_{19}O_4Cl$ MW 335 m/z

Table 3-11: Proposed Identification of DES and ESI Positive Ozonation Byproducts

Analyte	Retention Time (min)	Identity
DES	18 min	Diethylstilbestrol
Byproduct 9	7 min	MW 313 m/z
Byproduct 10	8 min	$C_{18}H_{19}O_4Cl$ MW 337 m/z
Byproduct 11	9 min	MW 353 m/z
Byproduct 12	10 min	$C_{18}H_{20}O_4$ MW 300 m/z
Byproduct 13	12 min	$C_{18}H_{20}O_4$ MW 300 m/z
Byproduct 14	13 min	$C_{18}H_{20}O_4$ MW 300 m/z
Byproduct 15	14 min	MW 241 m/z
Byproduct 16	15 min	$C_{18}H_{20}O_4$ MW 300 m/z
Byproduct 17	17 min	$C_{18}H_{20}O_4$ MW 300 m/z
Byproduct 18	19 min	$C_{18}H_{19}O_4Cl$ MW 337 m/z
Byproduct 19	21 min	$C_{18}H_{19}O_4Cl$ MW 337 m/z

Eight byproducts were formed during the ozonation of DES and detected in ESI negative mode. These byproduct formations were the result of oxygen radicals and molecular ozone attacking the phenolic moieties at the ortho position(s), with respect to the hydroxyl groups at C3 and C16. Based on the isotope pattern for byproducts 2, 8, 10, 18 and 19, chlorinated ozonation byproducts were produced from chlorine radicals which resulted from dilute levels of HCl (10^{-4} M) in the ozone stock solution. Byproducts 9, 11 and 15 were not identified due to the insufficient fragmentation information on the mass spectra.

The mass spectra for DES and each ozonation byproduct formed are located in Appendix B. The proposed reactions between ozone and DES are shown in Figure 3-58 and Figure 3-59, where

solid arrows indicate identified byproducts and dashed arrows signify potential byproducts. The chlorinated DES ozonation byproducts are given in Figure 3-60.

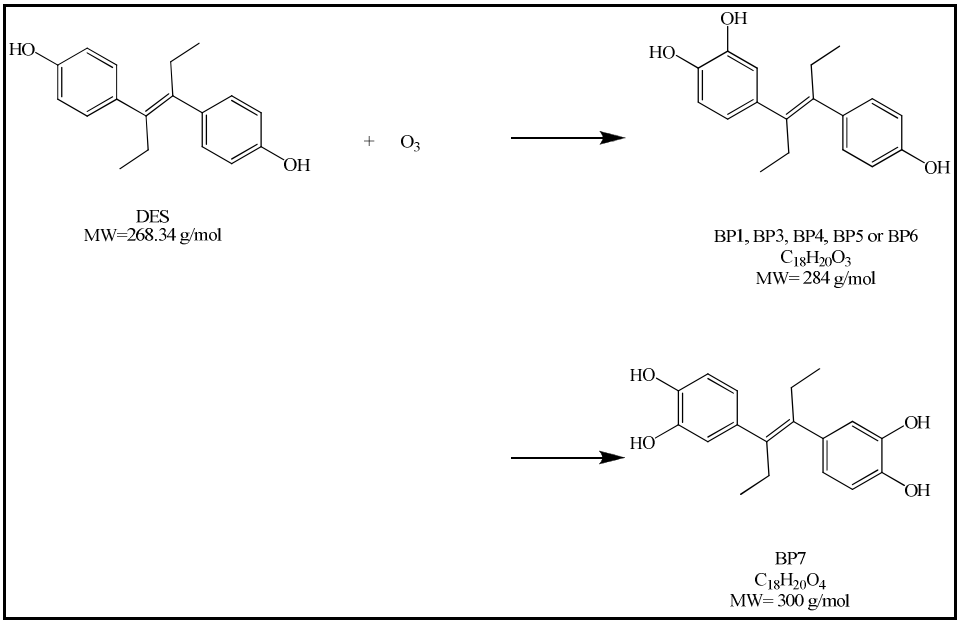


Figure 3-58: Proposed ESI Negative Byproducts from Reaction of O_3 and DES

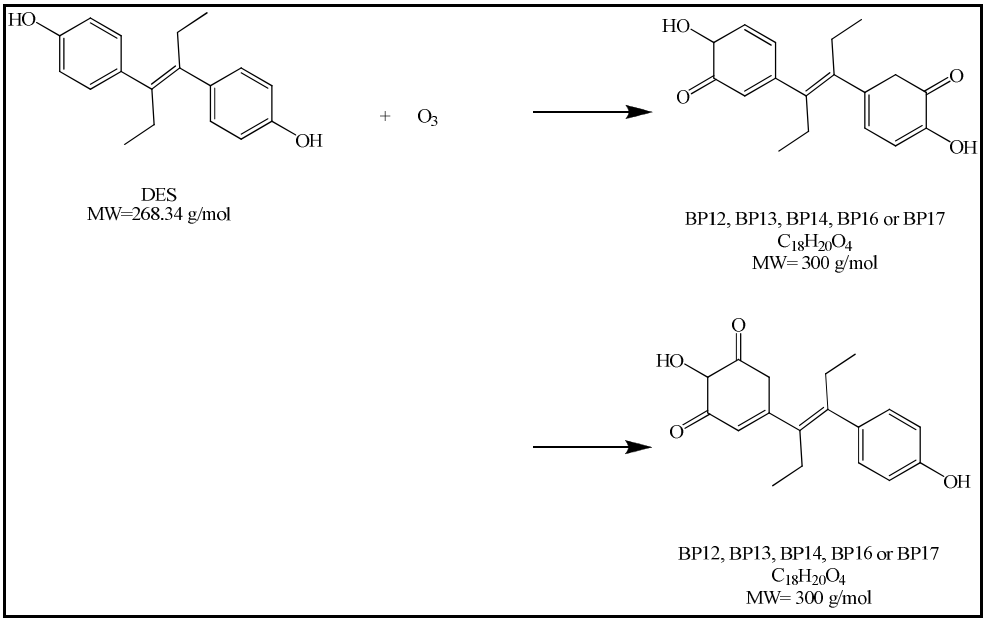


Figure 3-59: Proposed ESI Positive Byproducts from Reaction of O_3 and DES

A byproduct with the mass per charge of 285 m/z was found in the solvent front on the ESI positive spectra. Since this was discovered in the solvent front, the byproduct could not be isolated in terms of molecular weight or quantified with respect to DES.

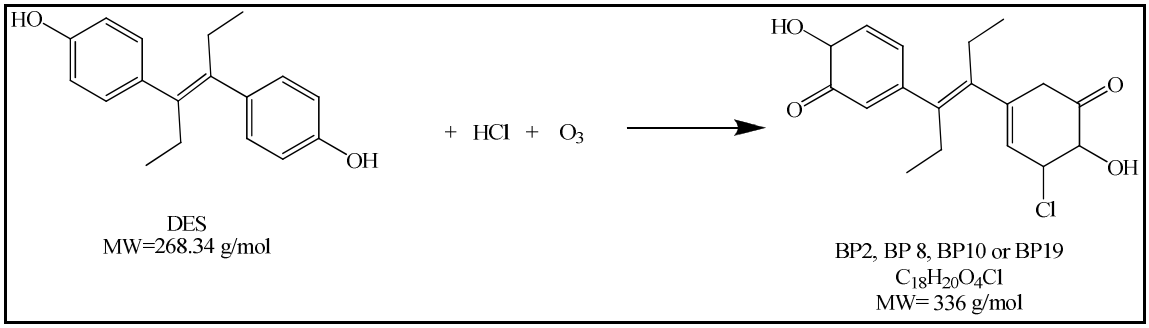


Figure 3-60: Proposed Chlorinated Ozonation Byproducts of DES

Relative Quantification

Since the ozone stock solution did not attain a highly concentrated solution, the short term ozonation dose volumes were relatively high compared to the other treatment dose volumes. This larger volume dose resulted in a dilution of the sample solutions and an artificial decrease in DES concentration for the short term reactions.

The relative concentration of each ozonation byproduct was determined based on peak areas since authentic standards were unavailable for individual byproducts. For purposes of relative concentrations, it was assumed that the only byproducts formed during the reactions were those that absorbed at a wavelength of 240 nm. The estimated quantification of each byproduct was based on the initial concentration of DES (10.0 ± 1.0 µM) and the molecular weights found for each byproduct. Figure 3-61 and Figure 3-62 provide DES and its ozonation (ESI negative and ESI positive) byproducts with respect to dose.

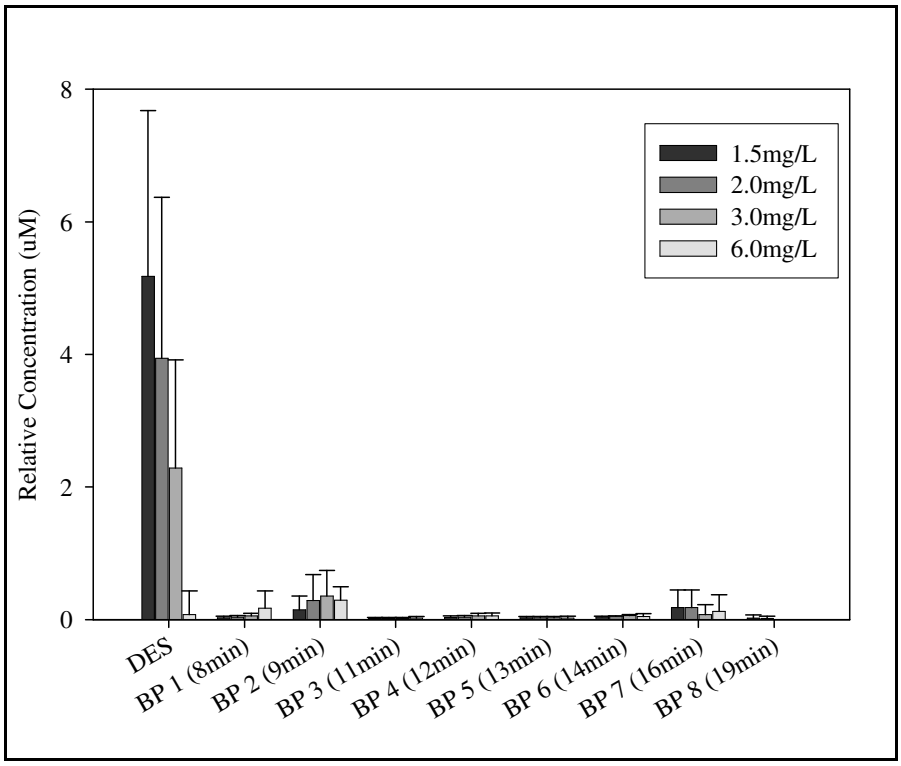


Figure 3-61: Relative Concentrations of DES Short Term ESI Negative Ozonation Byproducts

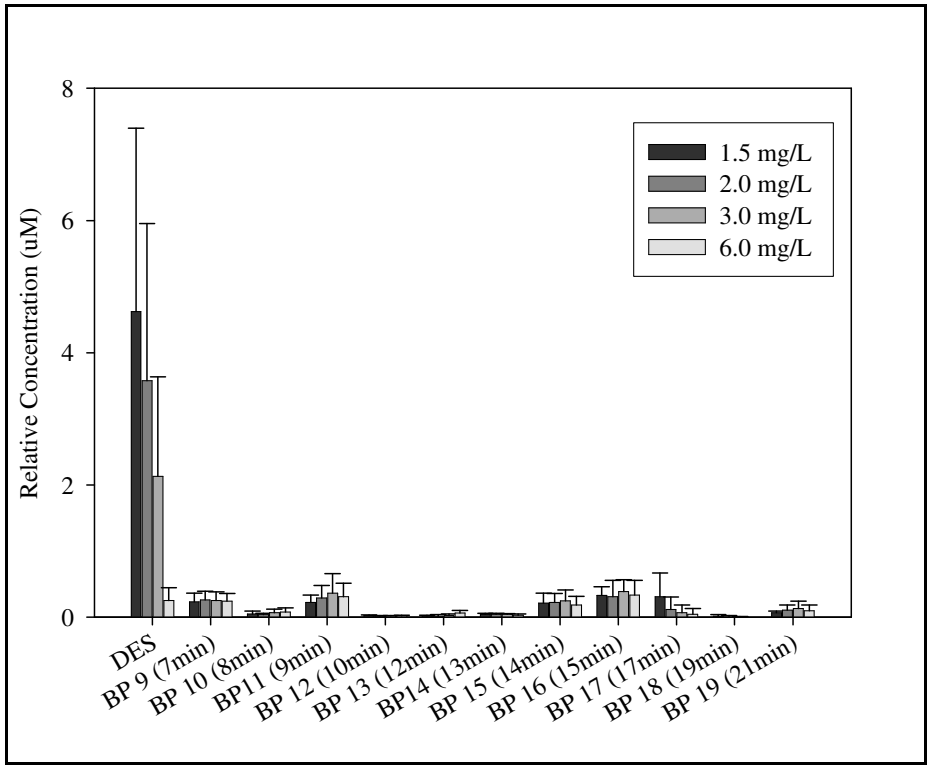


Figure 3-62: Relative Concentrations of DES Short Term ESI Positive Ozonation Byproducts

The instantaneous ozonation reactions showed a DES degradation of 35 % for 0.5 mg/L O_3 instantaneous dose. For the short-term reactions with a dose of 6.0 mg/L O_3, DES showed a percent degradation of 100 %.

3.6 Comparison of Disinfectant Treatments

Each treatment selected at least partially degraded the EDC contaminants; however, the efficacy of the treatment in reducing the parent compound showed a wide range with disinfection types.

Free chlorine disinfection treatment demonstrated the highest degradation potential for both EE2 and DES, as compared to chloramination and ozonation. Both EE2 and DES have multiple potential reactions sites with chlorine due to the resonance capabilities of the phenolic moieties and their electron donating potentials. The chlorination of EE2 resulted in nearly 100 % degradation for instantaneous, short term and long term reactions at the highest doses. The same number and amount of byproducts were observed for all three contact times. The DES and chlorine reaction showed 93 to 100 % degradation of the parent compound and a range of four to five byproduct formations directly correlating with the increased chlorine dose level.

The chloramination of EE2 and DES provided the lowest degradation for both EE2 and DES because of the lack of basic chloramine reaction sites on the acidic estrogenic compounds. The addition of ammonia after 30 sec of pre-chlorination effectively quenched the remaining free chlorine dose, which was also observed during the pre-ammoniation, simultaneous addition and pre-formed chloramines chloramination reactions. The pre-chlorination chloramination of EE2

showed a lower percent degradation as compared to the chlorination reactions, as well as a decreased number and quantity of byproducts with only 27 to 62 % degradation and two byproduct formations. These chloramination byproducts were identified as two of the three major byproducts associated with the chlorination of EE2; therefore, the predominant reaction during chloramination was chlorination of EE2.

The pre-chlorination chloramination of DES displayed a significantly lower degradation percentage, 18 to 55 %, as compared to the chlorination of DES. The instantaneous chloramination reactions did not produce any byproducts with the absorbance at 240 nm; however, the short term and long term reactions with DES produced two and three byproducts, respectively. Since the number of byproducts increased with the chloramination dose and the pre-chlorination chloramination of DES garnered the highest byproduct formations, the chlorination reaction was the predominant reaction within the system.

Because of the necessary dilution associated with each short term ozone dose, the percent degradation values were artificially lowered, thereby compromising the comparison of treatments. Even with this caveat, the degradation of EE2 during the short term ozone reaction was 97 % at 60 mg min/L. The degradation of DES was 100 % at 60 mg min/L O_3, which was expected due to the double phenolic moiety providing increased reaction sites as compared to other estrogenic EDCs. If the dose and parent degradation are compared, and the dilution is ignored, then ozonation of both EE2 and DES was more effective than chlorination and chloramination, even with the elongated reaction period. In comparing the number of

byproducts formed with the ring structure still intact, ozonation of both EE2 and DES produced the highest byproduct formation potential out of the three selected disinfecting agents.

The number of observable byproducts formed during ozonation was significantly higher than the number formed during chlorination and chloramination of EE2 and DES. Since the ozonation reactions had the potential of forming both additional hydroxyl groups and carbonyl groups on the phenolic moieties, as well as a larger number of potential reaction sites, then an increased number of potential byproducts had the possibility of forming. Following the other byproduct formations, it was assumed that as the dose increased, the byproduct formation potential would also increase. This correlation was observed in the ozonation reaction with EE2, in that four byproducts were formed during instantaneous ozonation and sixteen were formed during short term ozonation. With the ozonation of DES, however, the relationship between dose and number of byproducts remained constant. The instantaneous and short term ozonation of DES both produced nineteen byproducts. The increased number of reaction sites on DES accounts for the increased number of byproducts, as well as the same identity and number of byproducts formed during the both dose ratios.

The chlorination of EE2 and DES produced the highest percent degradation of the parent compound, with the least number of byproducts. Since the concentration of the byproducts can only be relative to the initial parent compound, the concentrations can only be estimated. The molecular weights for each byproduct have been identified; therefore, the relative concentration of each byproduct can be obtained. To determine the actual total concentrations for comparison, calibration curves of each byproduct would be required and then the sum of the total byproducts

could be determined. Due to the lack of authentic standards, the total byproduct concentration can only be estimated with respect to the initial parent compound, which cannot exceed 10 µM.

Table 3-12 and Table 3-13 present the byproduct comparison for EE2 and DES of treatment contact time and dose.

Table 3-12: Byproduct Comparison for EE2

Treatment	Contact Time	C·t* (mg min L^{-1})	Percent Degradation of EE2[#]	Number of Byproducts Produced	Estimated Quantification of Total Byproducts Produced[#]
Chlorination	Instantaneous	149	100 %	5	10 µM
	Short	1800	100 %	5	10 µM
	Long	17280	100 %	5	10 µM
Chloramination (Pre-Chlorination)	Instantaneous	149	27 %	2	2.7 µM
	Short	1800	52 %	2	5.2 µM
	Long	17280	61.5 %	2	6.2 µM
Ozonation	Instantaneous	0.5	23 %	4	2.3 µM
	Short	60	97 %	16	9.7 µM

*Instantaneous refers to 3600 sec reaction time, as reported in this table; Chloramination dose reported as Cl_2 with Cl_2:N of 4; Ozone reaction duration was assumed to be 10 min.
[#]Based on ESI Negative peak areas and byproducts with ring structures intact.

Table 3-13: Byproduct Comparison for DES

Treatment	Contact Time	C·t* (mg min L^{-1})	Percent Degradation of DES[#]	Number of Byproducts Produced	Estimated Quantification of Total Byproducts Produced[#]
Chlorination	Instantaneous	149	93 %	4	9.3 µM
	Short	1800	97 %	4	9.7 µM
	Long	17280	100 %	5	10 µM
Chloramination (Pre-Chlorination)	Instantaneous	149	18 %	0	1.8 µM
	Short	1800	37 %	2	3.7 µM
	Long	17280	55 %	3	5.5 µM
Ozonation	Instantaneous	0.5	35 %	19	3.5 µM
	Short	60	100 %	19	10 µM

*Instantaneous refers to 3600 sec reaction time, as reported in this table; Chloramination dose reported as Cl_2 with Cl_2:N of 4; Ozone reaction duration was assumed to be 10 min.
[#]Based on ESI Negative peak areas and byproducts with ring structures intact.

4 Conclusions

Emerging public health concerns relating to the epigenetic effects of EDCs, along with the reconceptualization of dose response curves, provides a compelling rationale for addressing estrogenically active contaminants in drinking water. These environmental health concerns are now known to have long lasting impacts, especially on fetal development.

For detection and quantification of EDCs, the optimized analytical method presented and implemented in this research was successfully used to determine the percent degradation of the parent compound for each disinfection treatment selected. With the use of the molar absorption coefficients and the absorbance chromatograms, the optimized method decreased sample variability between injections. The optimized method also provided a higher R^2 in the linear calibration with both high and low concentrations. Compared to the AquaAnalysis and EPA 1694 methods, the optimized method also lowered the method quantification limits and the method detection limits.

For this drinking water research, the estrogenic EDC byproducts were isolated and the treatment processes were compared using the dose applied, the number of byproducts formed and the relative quantification of the treatment byproducts. Various contact time intervals, including instantaneous, short and long term contact, were used to compare the treatments. The initial byproducts formed from the reactions were found with near instantaneous contact (on the order of seconds). To simulate the contact time used in drinking water treatment plants, a short contact time was selected for each type of treatment process, excluding chloramination because this treatment is typically used as a secondary disinfectant. Long contact times of 48 hours were

used to simulate the distribution system, with the exclusion of ozonation because this treatment is typically only used as a primary disinfectant.

Each treatment process selected at least partially degraded the EDC contaminants. The efficacy, however, of the respective treatments in reducing the parent compound showed a wide range of degradation by disinfection types. Five chlorination byproducts of EE2 were observed with nearly 100 % degradation; only four byproducts, however, have been previously reported. Two byproducts were isolated as a result of a chlorination reaction during the introduction of chloramines, when 61.5 % EE2 was degraded. There were sixteen byproducts isolated from the ozonation of EE2 after 97 % degradation. Previous ozonation studies have postulated the byproducts formed for the EE2 reactions; however, these hypothesized byproducts agree with those proposed identifications within this research. For the DES reactions, there were five byproducts isolated for chlorination with nearly 100 % degradation and three byproducts isolated for chloramination, after 55 % degradation. The ozonation of DES yielded nineteen byproducts, with nearly 100 % degradation of the parent compound. Since there was no prior research or data available on the DES byproducts, proposed identification comparisons could not be made.

From the resulting data, the chlorination of EE2 and DES produced the highest percent degradation of the parent compound, with the least number of byproducts for which the ring structures were still intact. The chloramination of EE2 and DES provided the lowest degradation for both EE2 and DES because of the lack of basic chloramine reaction sites on the acidic estrogenic compounds. The number of observable byproducts formed during ozonation was

significantly higher than the number formed during chlorination and chloramination of EE2 and DES.

Proposed byproduct identifications utilized: the byproduct polarity relative to the parent compound; the molecular weight and fragmentation pattern obtained from the mass spectrum; the type of disinfection agent; the isotope pattern observed on the mass spectrum; and the absorbance of the byproduct at the local absorbance maximum of the parent compound. The proposed byproduct structures for both the chlorination and chloramination treatments of EE2 included intact and semi-intact ring structures with free chlorine substitution on the phenolic moiety and five-carbon ring. The ozonation byproducts of EE2 appeared to retain the parent ring structure and aromatic-like absorbance characteristics, with hydroxyl and carbonyl group substitution on the phenolic moiety and the five-carbon ring. The proposed byproducts for the chlorination and chloramination of DES maintained the parent ring structure with free chlorine substitution on both phenolic moieties. The identifications proposed for the DES ozonation byproducts included hydroxyl and carbonyl substitutions on both phenolic moieties, while the phenolic rings remained intact.

5 Recommendations for Future Research

Based on the observed data, there are amendments to the optimized method that could enhance byproduct detection capabilities. Some byproducts could have been masked in the solvent front, providing a decreased number and quantity of byproducts produced from a given reaction. The LC mobile phase method would need to be amended to provide a longer duration with a lower ratio of organic solvent to water, so that the more polar compounds would have increased retention on the stationary phase. Another type of mobile or stationary phase could also be used to increase the retention times of compounds with high polarity, which may result in larger numbers of observable byproducts.

Changes to the treatment methods could also establish a more stable basis for treatment comparisons. The chlorination and chloramination reactions could be optimized for residual measurements at the shorter reaction times by using an in-line absorbance reaction cell. Another method for producing ozone stock solution is needed so that the dose volume is lowered and the temperature is closer to the sample temperature. These changes would decrease the method logically induced error discrepancies for the ozonation process as compared to the chlorination and chloramination processes.

Further research on the link between absorbance and the estrogenic strength of individual byproducts is needed to strengthen the optimized method with verification that the phenolic moiety is the binding site with the estrogen receptor. The use of nuclear magnetic resonance (NMR) spectroscopy of individual byproducts is necessary to verify each proposed byproduct structure, also confirming the intact ring structure for residual estrogenic behavior. After

positive identification of each byproduct, calibration curves need to be prepared for accurate concentration determination and signal amplification through MRMs.

Future research also needs to include other disinfection treatments (chlorine dioxide, UV irradiation, UV irradiation plus ozone, UV irradiation plus hydrogen peroxide, etc.) and other biologically active EDCs (natural estrogens, natural androgens, synthetic androgens, etc). The synergistic effect of these compounds also needs research attention, so that interactions between the biologically active compounds can be studied along with individual treatment effects. Continuing research is needed to help in understanding the complete consequences of estrogenic endocrine disruptors in drinking water and the inevitable public health impact.

Appendix A: Analytical Methods Section Support Data

Appendix A Figures

Appendix A Tables

AquaAnalysis Methods

Table A- 1: AquaAnalysis SPE Loading Method

Loading Method Solvent A: 100 % H_2O Solvent B: 100 % MeOH (wash solvent)				
Time (min)	**Flow (mL/min)**	**A%**	**%B**	**Curve**
Initial	5.00	100.0	0.0	Initial
0.20	4.00	80.0	20.0	11
4.00	1.00	100.0	0.0	11
12.00	4.00	100.0	0.0	11

Table A- 2: AquaAnalysis SPE Elution Method

Elution Method Solvent A: 2 % NH_4OH in H_2O Solvent B: 2 % NH_4OH in ACN				
Time (min)	**Flow (mL/min)**	**%A**	**%B**	**Curve**
Initial	0.40	70.0	30.0	Initial
3.00	0.40	70.0	30.0	6
12.50	0.40	35.0	65.0	6
13.00	0.40	35.0	65.0	6
14.00	0.40	70.0	30.0	6
15.00	0.40	70.0	30.0	6

Table A- 3: AquaAnalysis SPE Flushing and Reconditioning Method

Flushing and Reconditioning Method Solvent A: 100 % MeOH Solvent B: 20 % Acetone, 80% Ethyl Acetate Solvent C: 20 % Acetone, 80 % Pentane		
Time (min)	**Event**	**Action**
Initial	Set Flow A,B,C	0.10
2.50	Set Flow A	4.00
5.50	Set Flow B	2.00
5.50	Set Flow C	2.00
9.50	Set Flow C	0.10
9.50	Set Flow B	0.10
12.50	Set Flow A	0.10

Table A- 4: AquaAnalysis Autosampler Method

Autosampler Method	
Repeat Injections	1
Syringe Volume	5.0 mL

Table A- 5: AquaAnalysis MS/MS Method Optimizations

MS Method	
Ionization Mode	ESI-
Number of MRMs	5 pairs
Time	0.00 to 15.00 min
Inter-Channel Delay	0.02
Inter-Scan Delay	0.02
Repeats	1
Span	0.1

Table A- 6: AquaAnalysis MRM Optimizations for Estrogenic EDCs

Analyte	**Parent (m/z)**	**Daughter (m/z)**	**Dwell (sec)**	**Cone (V)**	**Collision Energy (eV)**
Estrone	269.3	145.3	0.2	50	30
Estradiol	271.3	145.3	0.2	50	35
Estriol	287.1	171.0	0.2	55	35
17β-Ethinyl Estradiol	295.2	145.2	0.2	50	40
Diethylstilbestrol	267.1	251.2	0.2	40	25

Table A- 7: AquaAnalysis MS Tune Parameters

MS Tune	
ES-Source	
Capillary	3.50 kV
Cone	45 V
Exactor	3 V
RF Lens	0.3 V
Source Temp	140°C
Desolvation Temp	350°C
Desolvation Gas Flow	550 L/hr
Cone Gas Flow	50 L/hr
Analyser	
LM Resolution 1	13.0
HM Resolution 1	13.0
Ion Energy 1	0.4
Entrance	-2
Collision	40
Exit	0
LM Resolution 2	13.0
HM Resolution 2	13.0
Ion Energy 2	0.8
Multiplier	650
API Gas	UHP Nitrogen
Collision Gas	UHP Argon

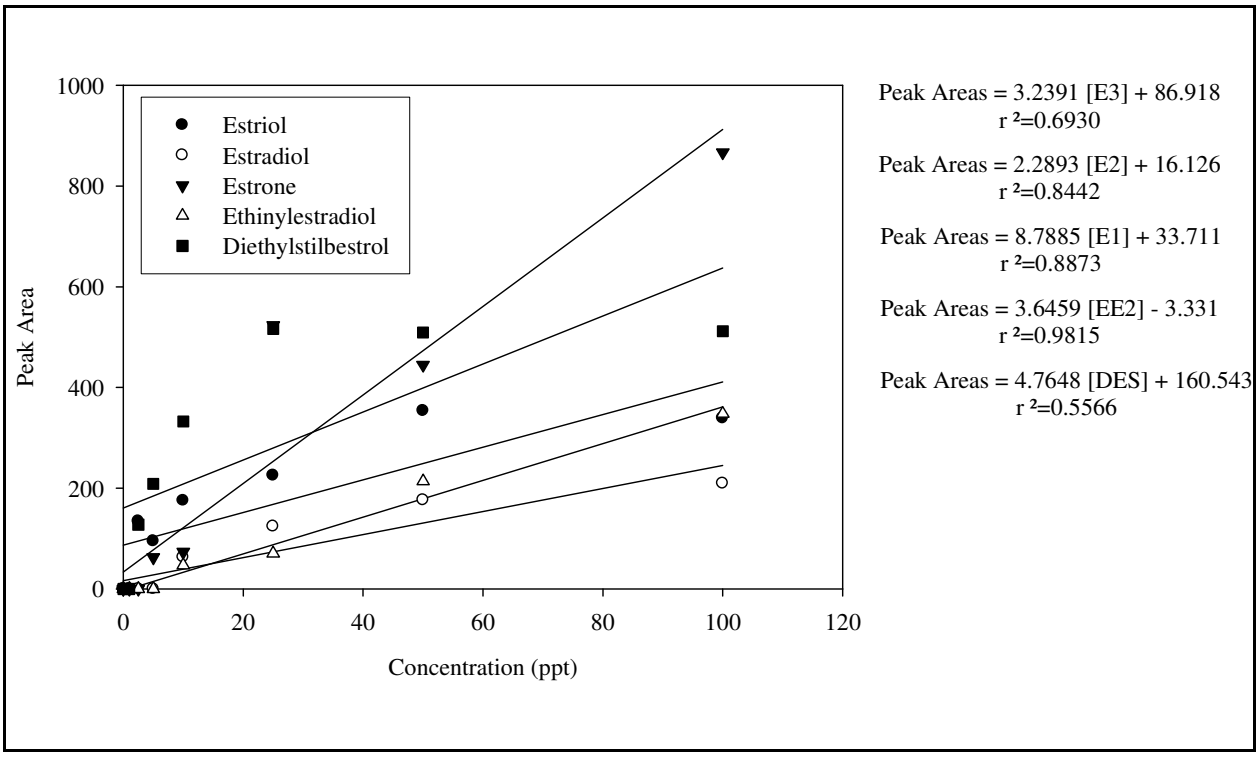


Figure A- 1: AquaAnalysis Low Concentration Curves

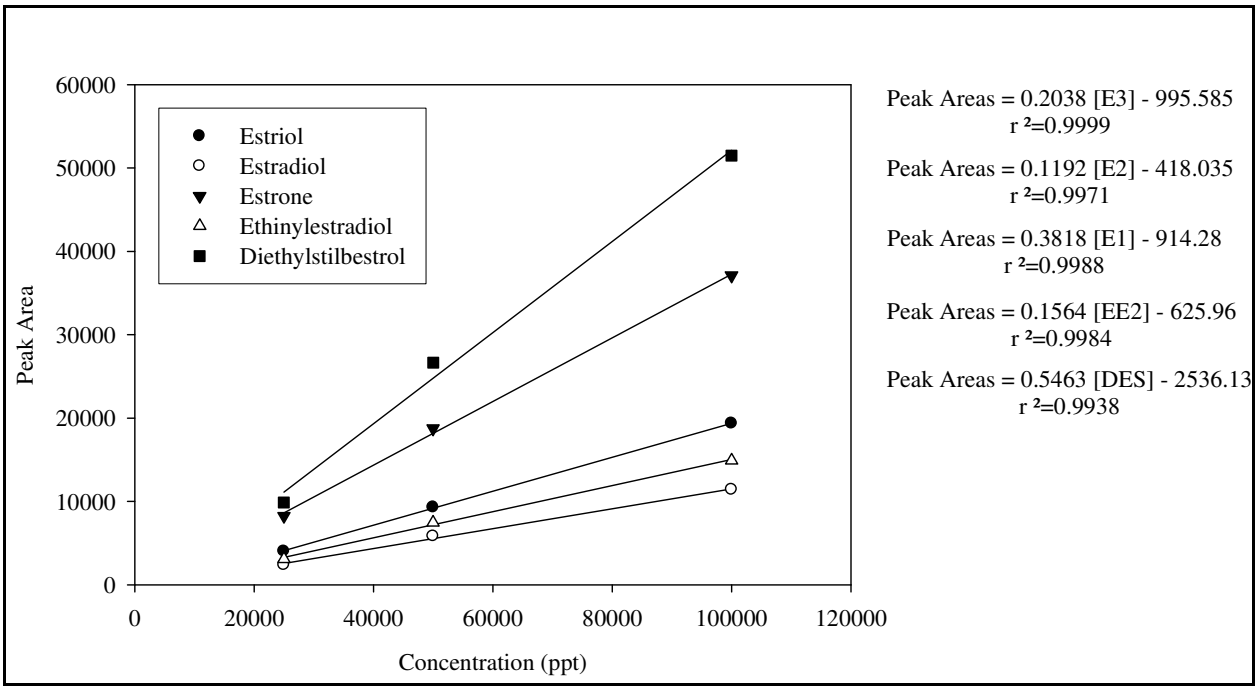


Figure A- 2: AquaAnalysis High Concentration Curves

EPA 1694 Method

Table A- 8: EPA 1694 SPE Conditions

SPE: Extraction and Concentration Conditions	
Solid Phase	HLB Oasis® Cartridge
Cartridge Conditioning	20 mL Methanol and 6 mL Water
Sample Loading Rate	5-10 mL/min
Extraction Volume	1 L
Column Washing	10 mL Water
Column Drying Time	5 min
Column Elution	12 mL Methanol
Blow-Down Gas	UHP Nitrogen
Water Bath Temperature	50 ± 5 °C
Solvent Addition	3 mL Methanol
Final Extract Volume	4.0 ± 0.1 mL with 0.1% Formic Acid Solution

Table A- 9: EPA 1694 General LC Conditions

General LC Conditions	
Instrument	Waters Alliance 2695 Separation Module with Waters Quattro *micro*™ API Quadrupole Mass Spectrometer
LC Column	Waters C-18 Atlantis® T3, 3µm 2.1x50mm
Ionization	ESI-
Acquisition	MRM mode, unit resolution
Injection Volume	15 µL
Column Temperature	40 °C
Flow Rate	0.200 mL/min
Max Pressure	345 Bar
Autosampler-Tray Temperature	4 °C
LC Solvent	Solvent A: 0.1 % Ammonium Acetate and 0.1 % Acetic Acid in HPLC water Solvent B: 1 to 1 Methanol to Acetonitrile

Table A- 10: EPA 1694 LC Gradient Program

LC Gradient Program		
Time (min)	**Flow Mixture**	**Gradient**
0.0	60 % Solvent A 40 % Solvent B	1
0.5	60 % Solvent A 40 % Solvent B	6
7.0	100 % Solvent B	6
12.5	100 % Solvent B	6
12.7	60 % Solvent A 40 % Solvent B	6
16.0	60 % Solvent A 40 % Solvent B	1

Table A- 11: EPA 1694 Method MS/MS Method Parameters

MS Method	
Ionization Mode	ESI-
Number of MRMs	5 pairs
Time	0.00 to 16.00 min
Inter-Channel Delay	0.02
Inter-Scan Delay	0.02
Repeats	1
Span	0.1

Table A- 12: EPA 1694 MRM Optimizations for Estrogenic EDCs

Analyte	Parent (m/z)	Daughter (m/z)	Dwell (sec)	Cone (V)	Collision Energy (eV)
Estrone	269.3	145.3	0.2	50	30
Estradiol	271.3	145.3	0.2	50	35
Estriol	287.1	171.0	0.2	55	35
17β-Ethinyl Estradiol	295.2	145.2	0.2	50	40
Diethylstilbestrol	267.1	251.2	0.2	40	25

Table A- 13: EPA 1694 MS Conditions

MS Conditions	
ES-Source	
Capillary	3.50 kV
Cone	45 V
Exactor	3 V
RF Lens	0.3 V
Source Temperature	100 ^{o}C
Desolvation Temperature	350 ^{o}C
Cone Gas Flow Rate	50 L/hr
Desolvation Gas Flow Rate	300 L/hr
Analyser	
LM Resolution 1	13.0
HM Resolution 1	13.0
Ion Energy 1	0.4
Entrance	-2
Collision	40
Exit	0
LM Resolution 2	13.0
HM Resolution 2	13.0
Ion Energy 2	0.8
Multiplier	650
API Gas	UHP Nitrogen
Collision Gas	UHP Argon

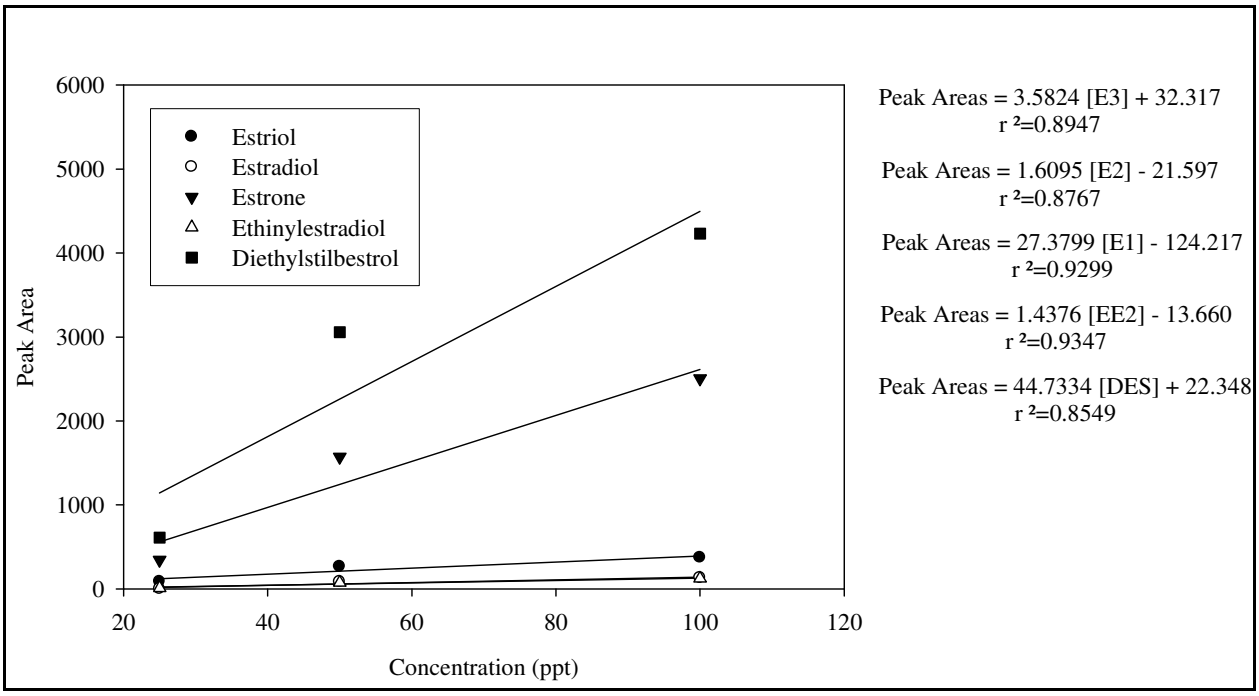


Figure A- 3: EPA 1694 Method Low Concentration Curves

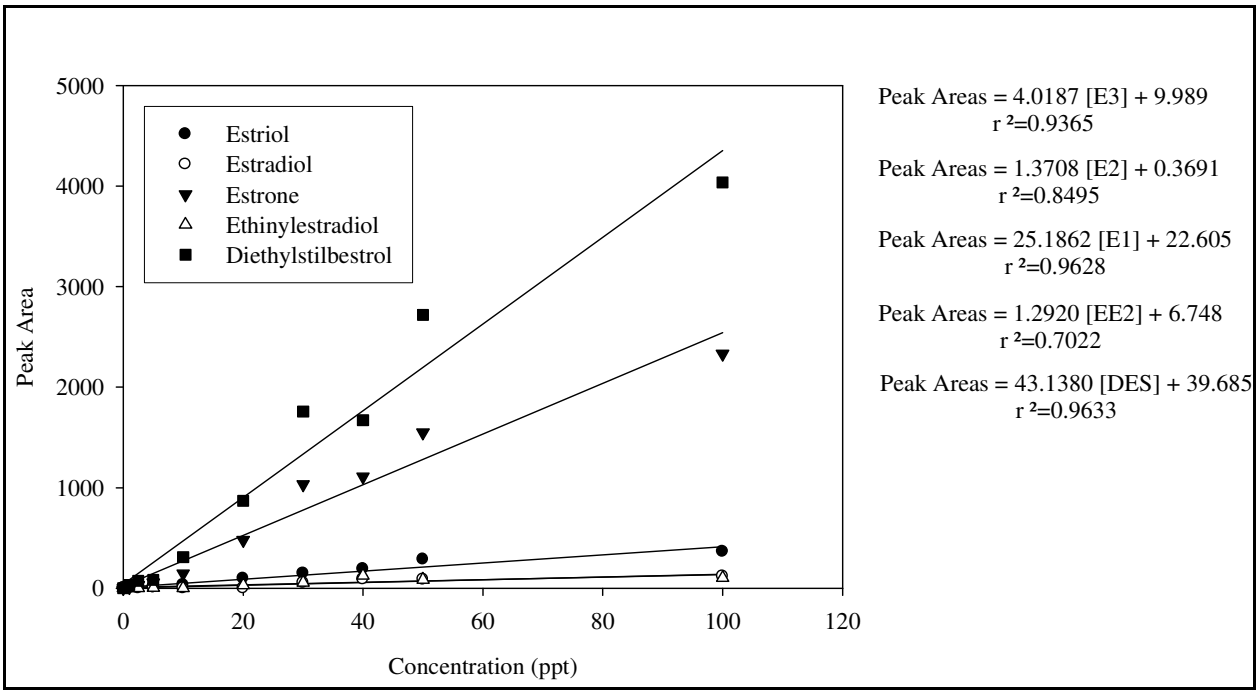


Figure A- 4: EPA 1694 Method High Concentration Curves

Optimized Method

Table A- 14: Optimized Method LC and PDA Conditions

General LC Conditions	
Instrument	Waters Alliance 2695 Separation Module with Waters Quattro *micro*™ API Quadrupole Mass Spectrometer
LC Column	Kromasil® C-18, 3.5µm 4.6x150mm
Ionization	ESI-
Acquisition	MRM mode, unit resolution
Injection Volume	100 µL
Column Temperature	40 °C
Flow Rate	0.500 mL/min
Max Pressure	345 Bar
Autosampler-Tray Temperature	4 °C
LC Solvent	Solvent A: Water Solvent B: Methanol
PDA Conditions	
Wavelength Range	210 to 400 nm
Resolution	1.2 nm
Sampling Rate	2 spectra/sec

Table A- 15: Optimized Method LC Gradient Program

LC Gradient Program		
Time (min)	**Flow Mixture**	**Gradient**
0.0	45 % Solvent A 55 % Solvent B	1
1.00	45 % Solvent A 55 % Solvent B	1
16.00	30 % Solvent A 70 % Solvent B	5
21.00	30 % Solvent A 70 % Solvent B	1
23.00	45 % Solvent A 55 % Solvent B	6

Table A- 16: Optimized Method MS/MS Method Parameters

MS Method	
Ionization Mode	ESI-
Number of MRMs	5 pairs
Time	0.00 to 23.00 min
Inter-Channel Delay	0.02
Inter-Scan Delay	0.02
Repeats	1
Span	0.1

Table A- 17: Optimized Method MRM Parameters for Estrogenic EDCs

Analyte	Parent (m/z)	Daughter (m/z)	Dwell (sec)	Cone (V)	Collision Energy (eV)
Estrone	269.3	145.3	0.2	50	30
Estradiol	271.3	145.3	0.2	50	35
Estriol	287.1	171.0	0.2	55	35
17β-Ethinyl Estradiol	295.2	145.2	0.2	50	40
Diethylstilbestrol	267.1	251.2	0.2	40	25

Table A- 18: Optimized Method MS Conditions

MS Conditions	
ES-Source	
Capillary	3.50 kV
Cone	45 V
Exactor	3 V
RF Lens	0.3 V
Source Temperature	140 ^{o}C
Desolvation Temperature	350 ^{o}C
Cone Gas Flow Rate	50 L/hr
Desolvation Gas Flow Rate	550 L/hr
Analyser	
LM Resolution 1	13.0
HM Resolution 1	13.0
Ion Energy 1	0.4
Entrance	-2
Collision	35
Exit	0
LM Resolution 2	13.0
HM Resolution 2	13.0
Ion Energy 2	0.8
Multiplier	650
API Gas	UHP Nitrogen
Collision Gas	UHP Argon

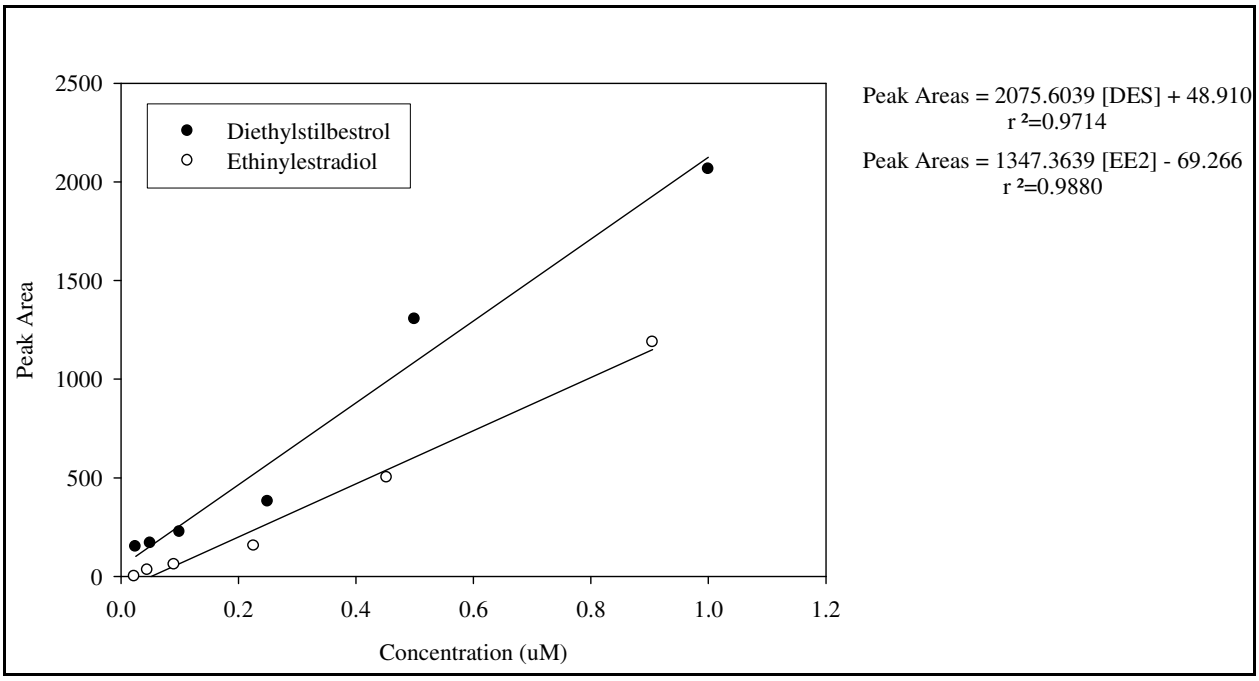


Figure A- 5: Optimized Method Low Concentration Curves

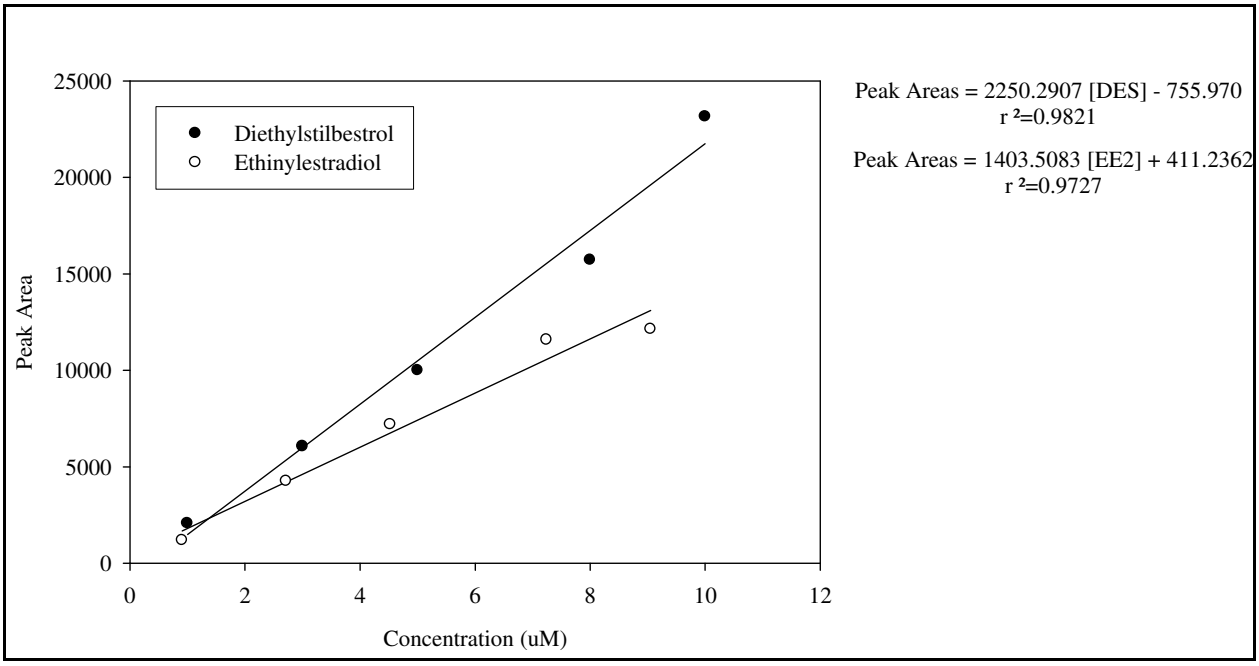


Figure A- 6: Optimized Method High Concentration Curves

Appendix B: EDC Treatment Section Support Data

Appendix B Figures

Appendix B Tables

Mass Spectra of Parent Compounds

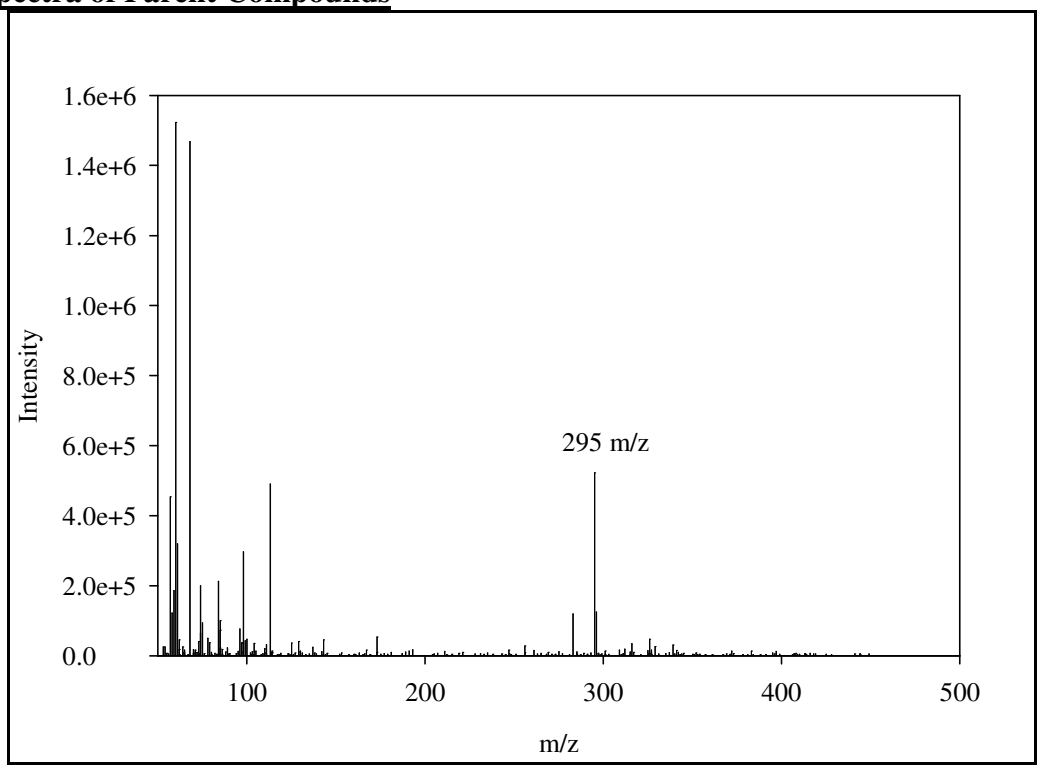


Figure B-1: Ethinylestradiol Mass Spectrum

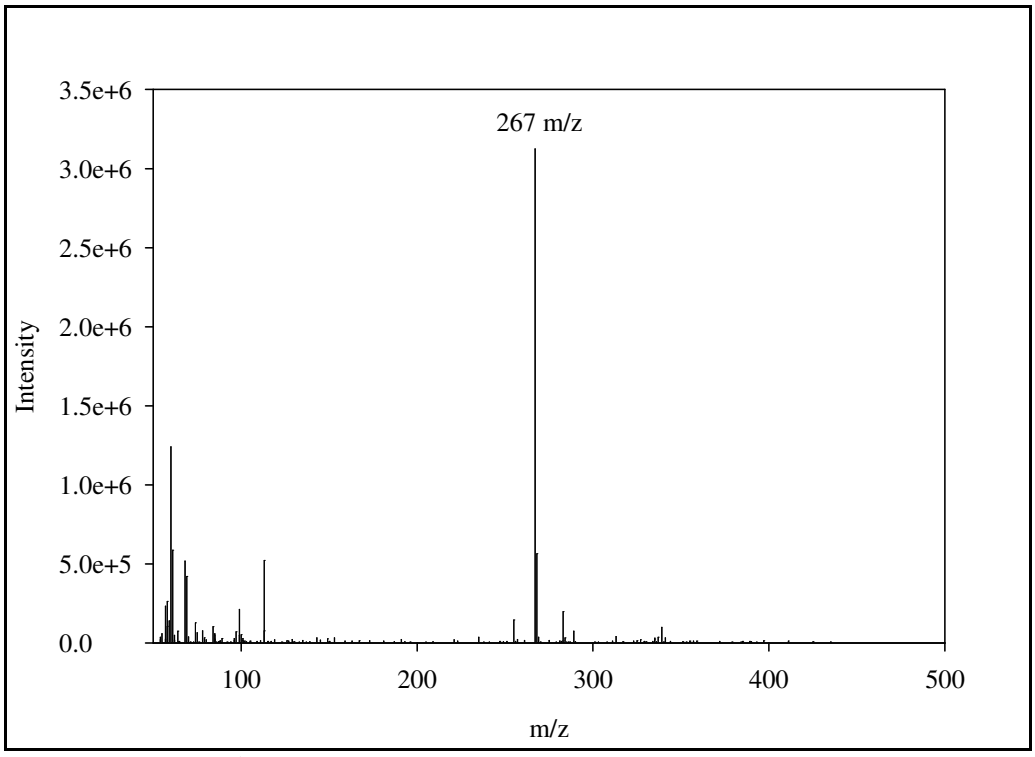


Figure B-2: Diethylstilbestrol Mass Spectrum

EE2 and DES Chlorination Methods
Initial ED concentrations of 10 ± 0.1 uM
Total chlorine concentrations of _____ uM
Temperature of solution 20 ºC

Sample times
0, 10, 20, 30, 40, 50, 60, 120, 300sec (5min), 600sec (10min), 1800sec (30min)

1) Sample size of 150 mL of 10 ± 0.1 uM EE2 with a pH adjustment to ____ with 10 mM phosphoric acid buffer.

2) Inject _____mL of chlorine solution for final concentration of 35 uM. (Cl solution should be approximately 1750 uM). Mix solution thoroughly.

3) At time intervals of 0, 10, 20, 30, 40, 50, 60, 120, 300, 600sec, inject 100uL of sodium thiosulfate solution (100g/L) to quench the residual chlorine and stop the oxidation reaction. Measure pH after reaction.

5) Samples analyzed using HPLC to determine the remaining ED concentration.

After degradation has occurred, the byproducts will need to be identified to determine if the estrogenic activity has increased or decreased for the new daughter compounds.

EE2 Chlorination

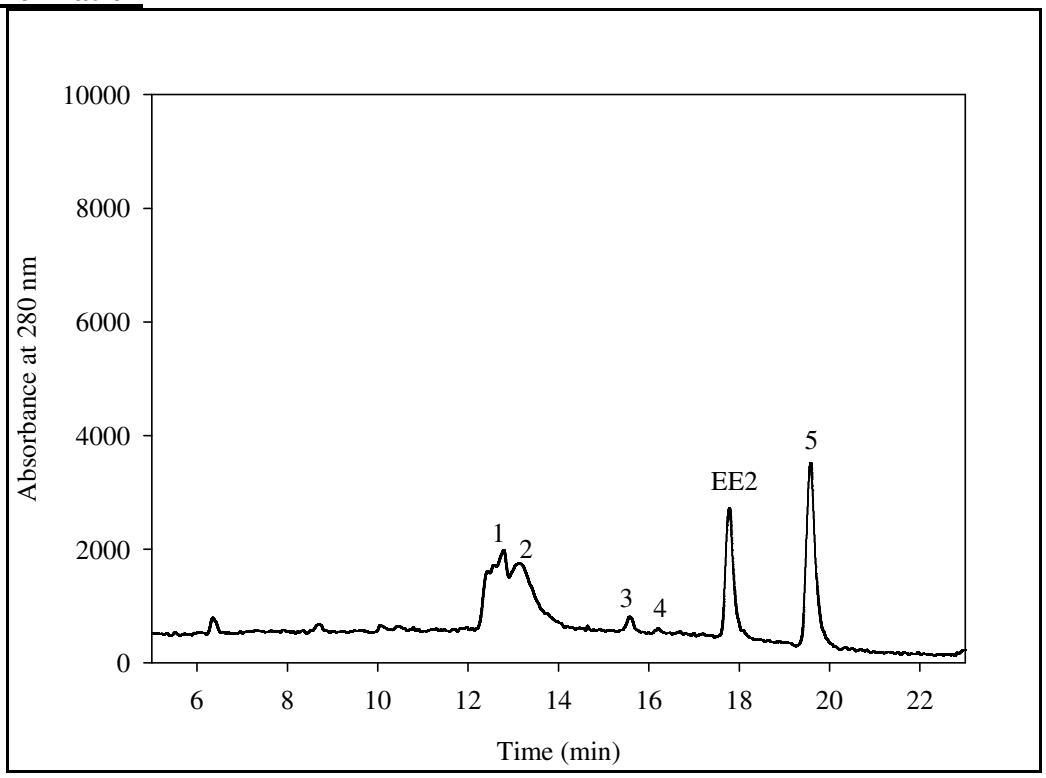


Figure B-3: Short Term Chlorination of EE2 Chromatogram

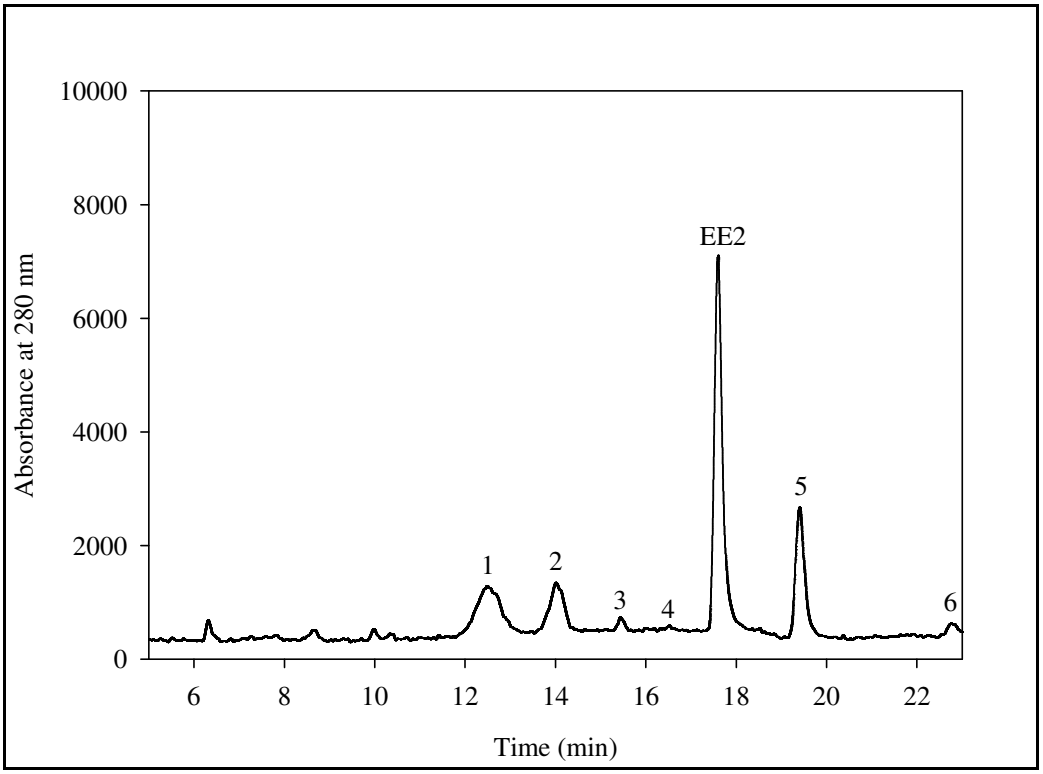


Figure B-4: Instantaneous Chlorination of EE2 Chromatogram

Table B-1: Instantaneous Chlorination of EE2

	% Total Peak Areas											
	0 sec	**10 sec**	**20 sec**	**30 sec**	**40 sec**	**50 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
EE2	100	93.4	74.0	67.9	61.9	50.8	49.6	22.6	4.4	1.7	0.0	0.0
Byproduct 1 (11min)	0	3.9	11.6	11.9	17.3	15.8	18.8	16.5	8.3	1.6	0.0	0.0
Byproduct 2 (19min)	0	1.3	9.3	13.3	12.2	21.5	18.4	39.9	60.2	66.7	64.4	65.2
Byproduct 3 (15min)	0	0.0	0.6	1.1	0.7	2.2	1.6	3.2	4.3	5.8	28.0	28.4
Byproduct 4 (16min)	0	0.0	0.0	0.0	0.2	0.0	0.3	0.8	0.4	0.0	4.4	5.2
Byproduct 5 (13min)	0	1.4	4.5	5.8	7.8	9.8	11.2	17.1	22.4	24.2	3.2	1.2
Other Byproducts	0	6.8	16.3	21.8	11.3	13.2	20.7	17.7	28.2	37.8	38.4	50.3

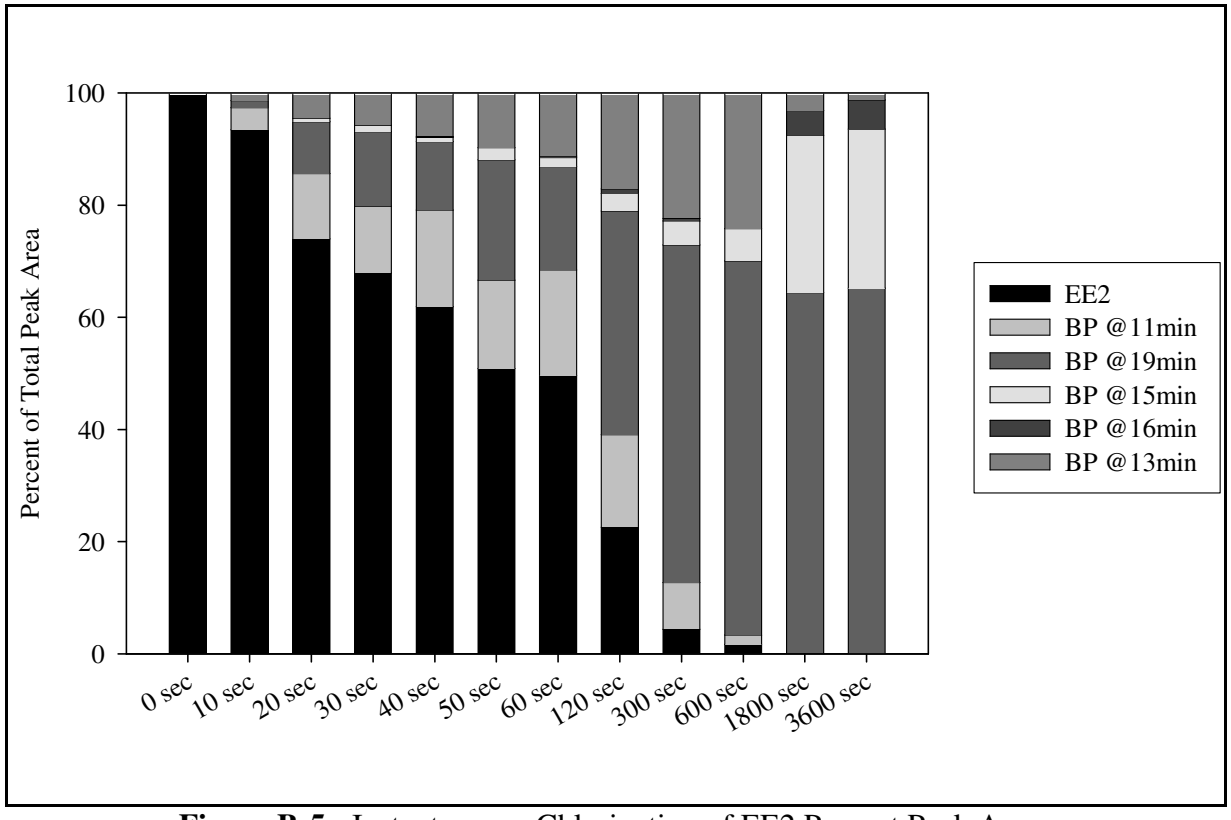


Figure B-5: Instantaneous Chlorination of EE2 Percent Peak Areas

Table B-2: Long and Short Term Chlorination of EE2

	Dose (mg/L)	Cl_2 Residual (mg/L)	Standard Deviation Cl_2 Residual	Concentration of EE2 (mg/L)	Standard Deviation of EE2
Short Term (5 hrs)	1.5	0	0	0.104	0.038
	3.0	0.455	0.047	0.003	0.003
	6.0	2.585	0.031	0.001	0.001
Long Term (48 hrs)	1.5	0	0	0.132	0.042
	3.0	0	0	0.003	0.002
	6.0	0.125	0.038	NDL	--

*NDL = non-detectable level

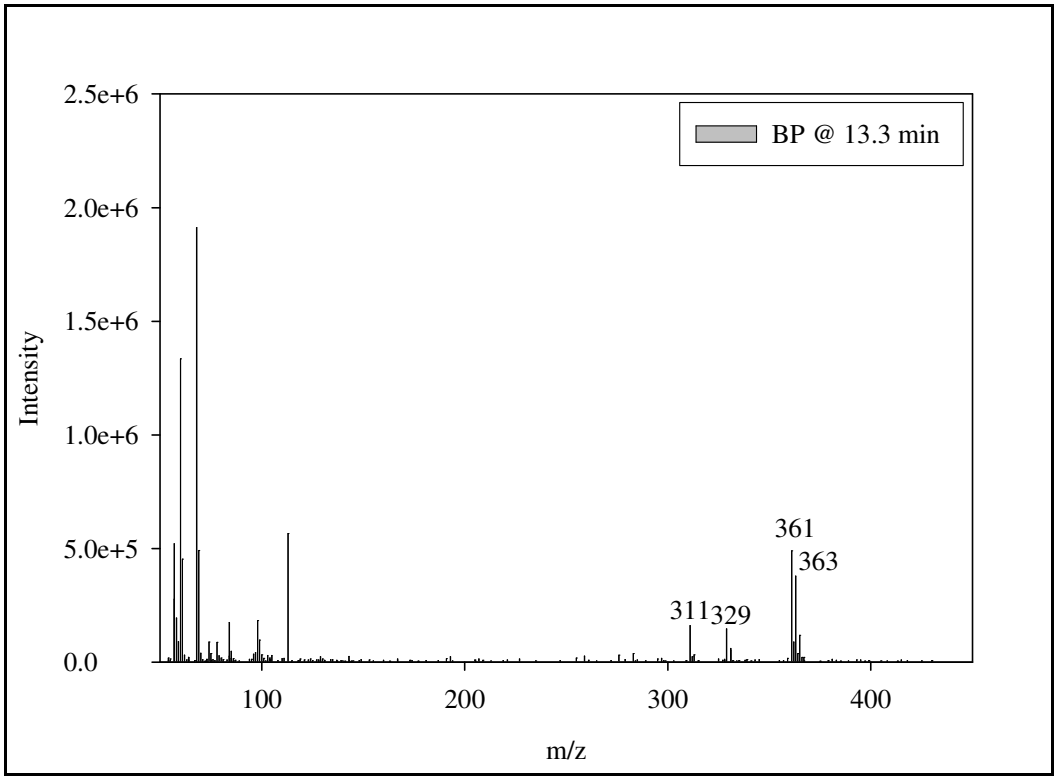


Figure B-6: Chlorination Byproduct of EE2

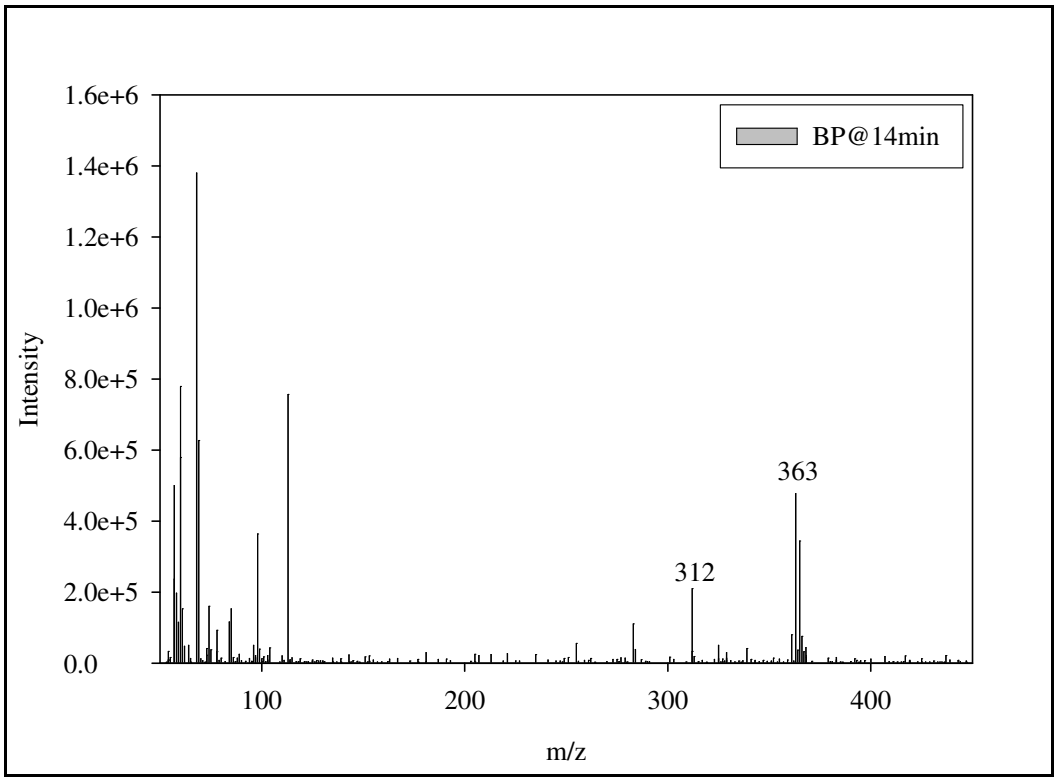


Figure B-7: Chlorination Byproduct of EE2

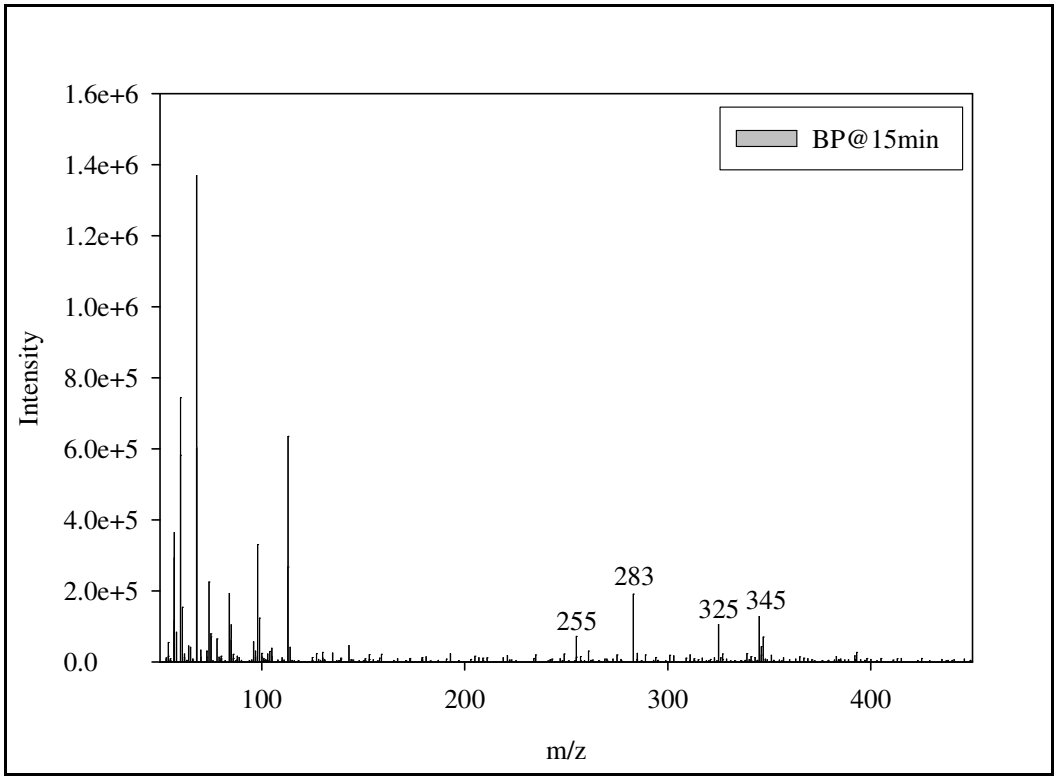


Figure B-8: Chlorination Byproduct of EE2

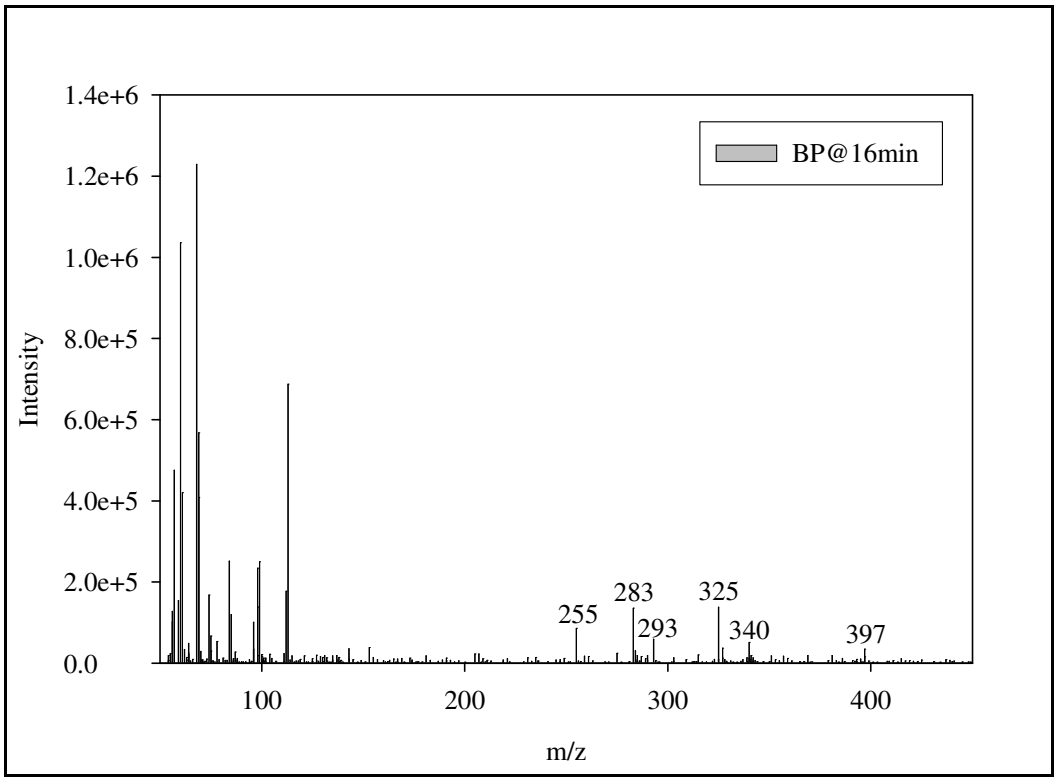


Figure B-9: Chlorination Byproduct of EE2

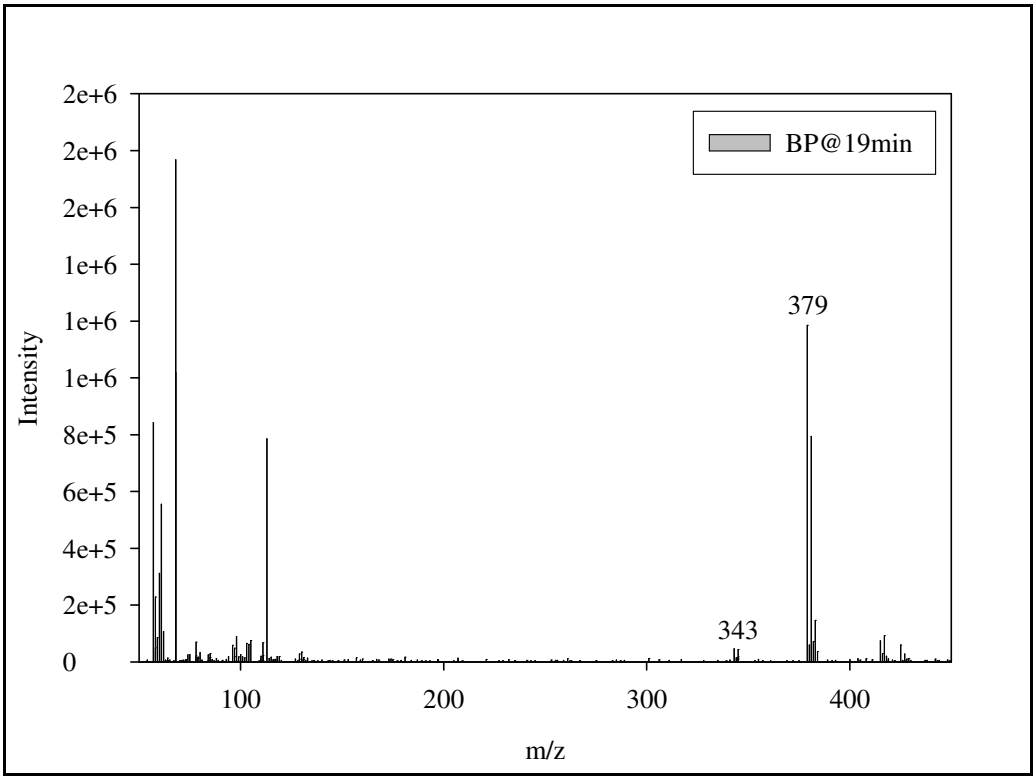


Figure B-10: Chlorination Byproduct of EE2

DES Chlorination

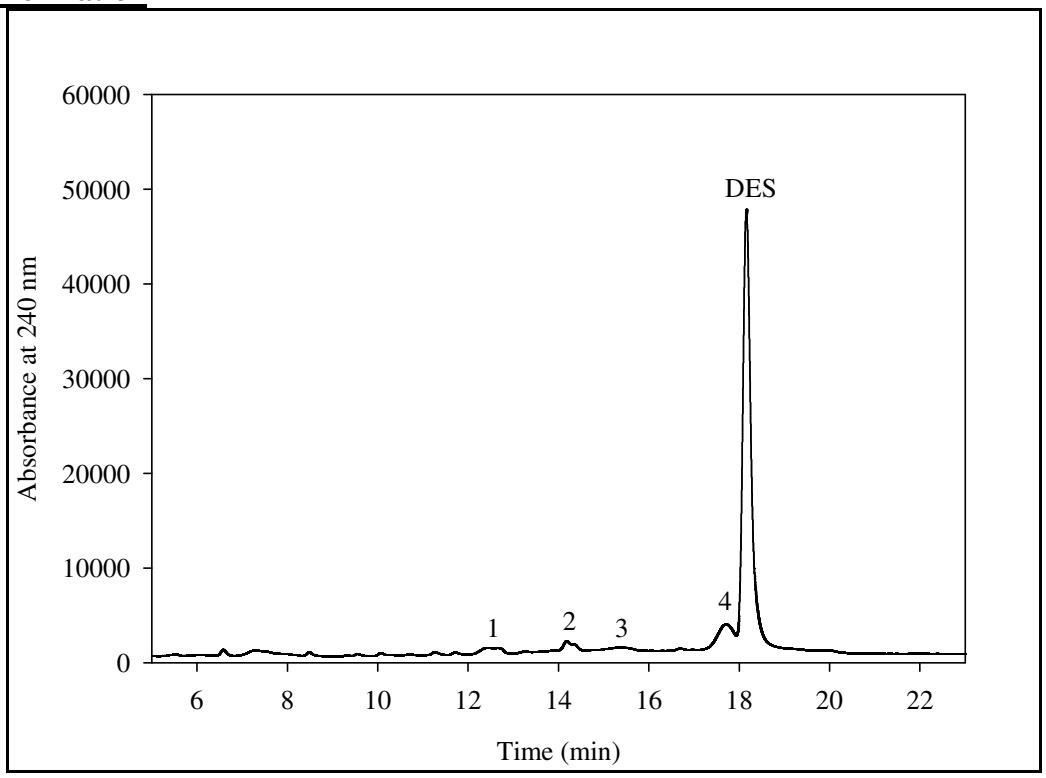


Figure B-11: Instantaneous Chlorination of DES Chromatogram

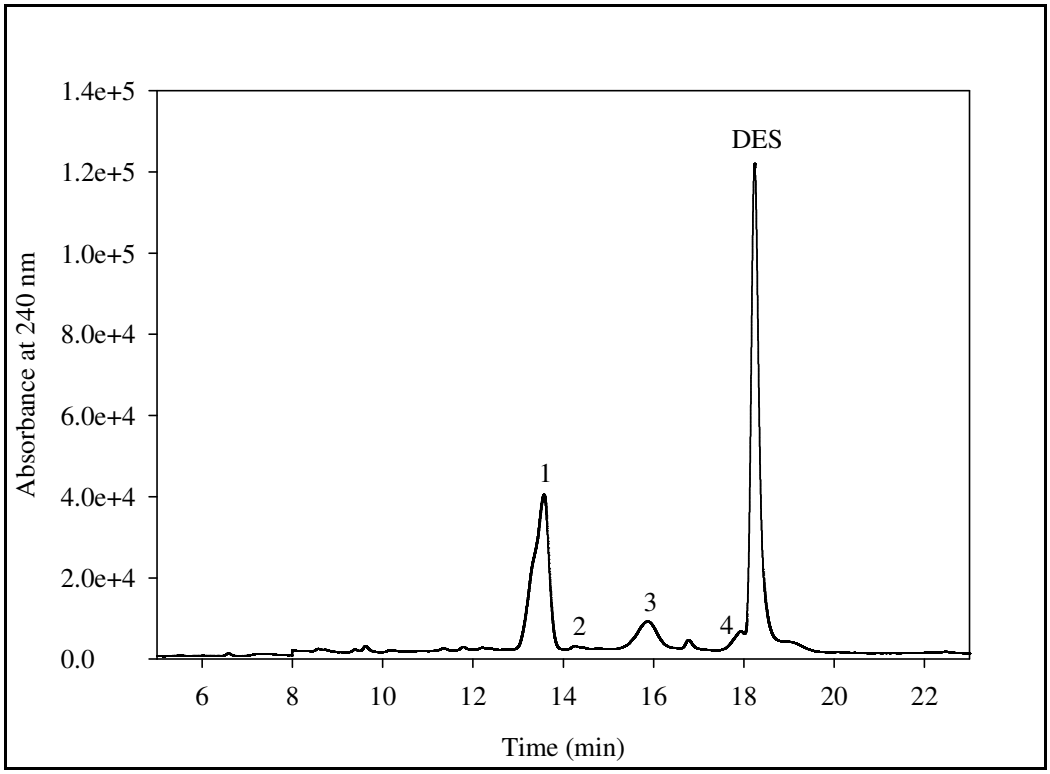


Figure B-12: Short Term Chlorination of DES Chromatogram

Table B-3: Instantaneous Chlorination of DES

	% Total Peak Areas											
	0 sec	**10 sec**	**20 sec**	**30 sec**	**40 sec**	**50 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
DES	97.8	96.4	91.5	87.0	83.6	78.9	78.5	58.8	30.3	13.1	2.4	0.6
BP 1 (13min)	0.0	0.0	0.0	0.0	1.3	2.0	2.6	7.5	20.0	31.2	29.5	24.6
BP 2 (14min)	2.2	2.1	2.1	1.9	1.7	2.6	2.4	2.2	2.0	1.1	0.4	0.0
BP 3 (15min)	0.0	0.0	0.0	0.1	0.1	1.3	1.6	2.3	5.1	7.4	5.9	5.2
Other BPs	0.0	1.5	6.4	11.1	13.3	15.3	14.9	29.2	42.5	47.3	61.8	69.6

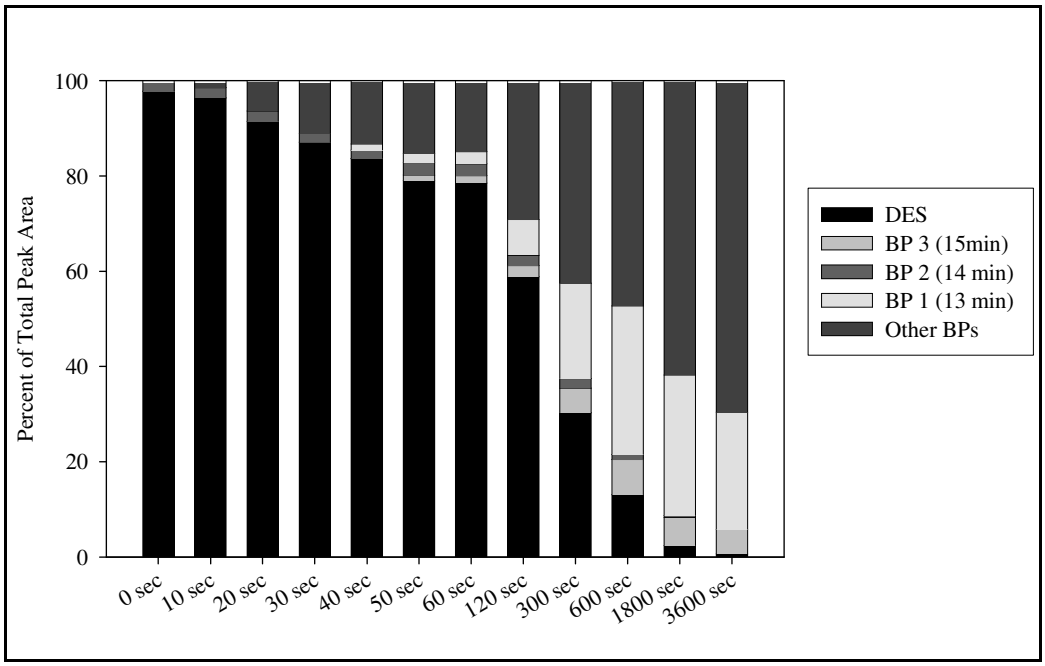


Figure B-13: Instantaneous Chlorination of DES Percent Peak Area

Table B-4: Long and Short Term Chlorination of DES

	Dose (mg/L)	Cl_2 Residual (mg/L)	Standard Deviation Cl_2 Residual	Concentration of EE2 (mg/L)	Standard Deviation of EE2
Short Term (5 hrs)	1.5	0	0	0.332	0.163
	3.0	0	0	0.022	0.002
	6.0	0	0	0	0.077
Long Term (48 hrs)	1.5	0	0	0.173	0.331
	3.0	0.018	0.013	0.003	0.007
	6.0	0.038	0.010	NDL	--

*NDL = non-detectable level

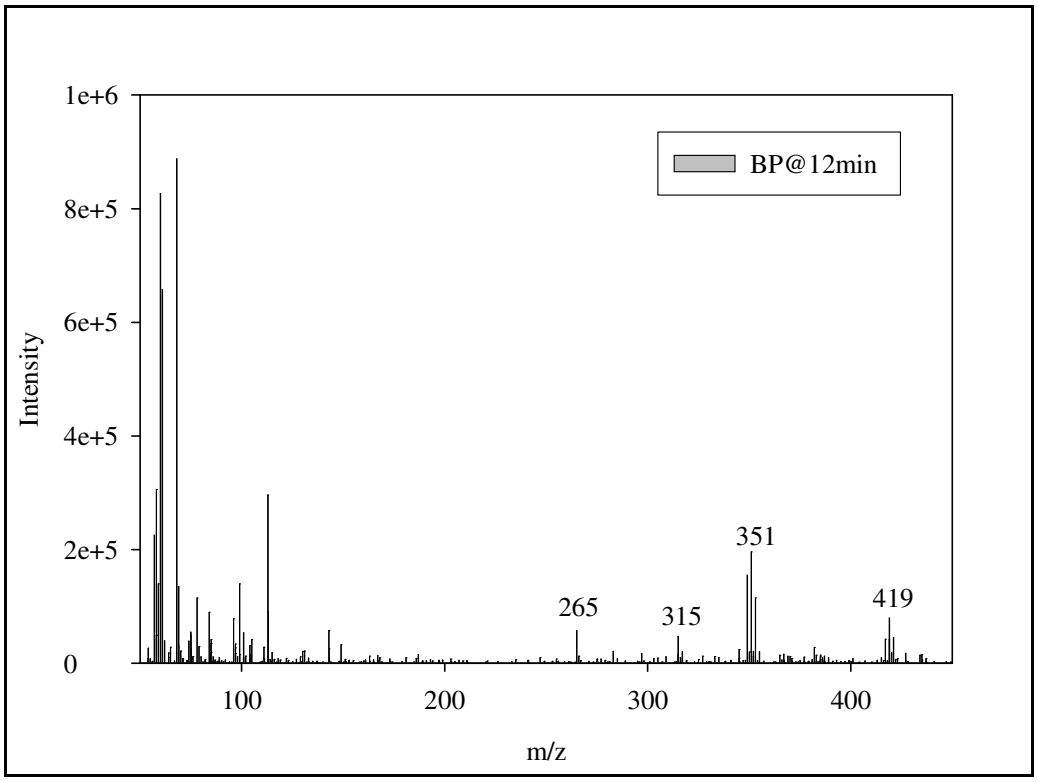


Figure B-14: Chlorination Byproduct of DES

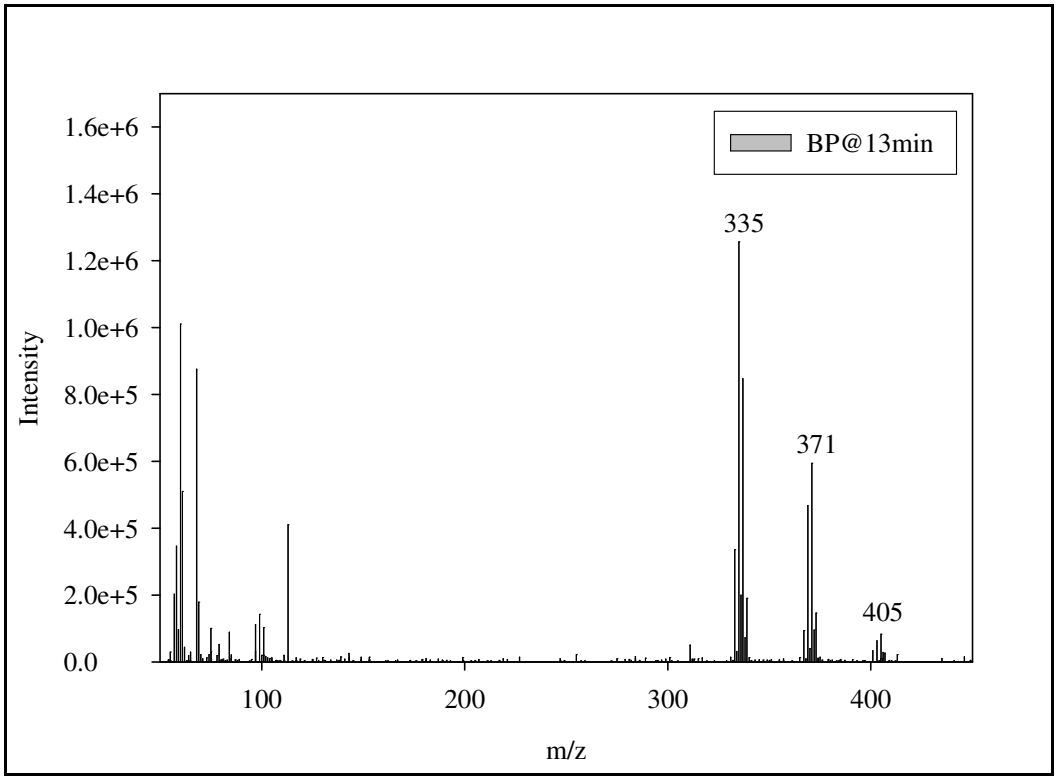


Figure B-15: Chlorination Byproduct of DES

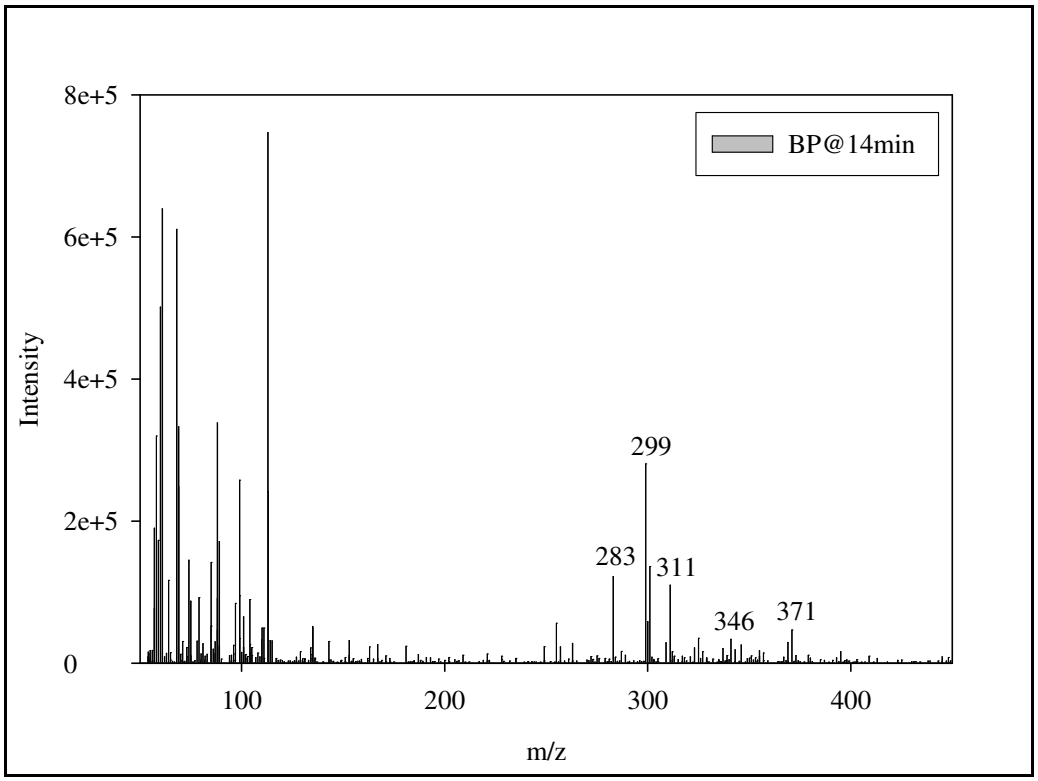


Figure B-16: Chlorination Byproduct of DES

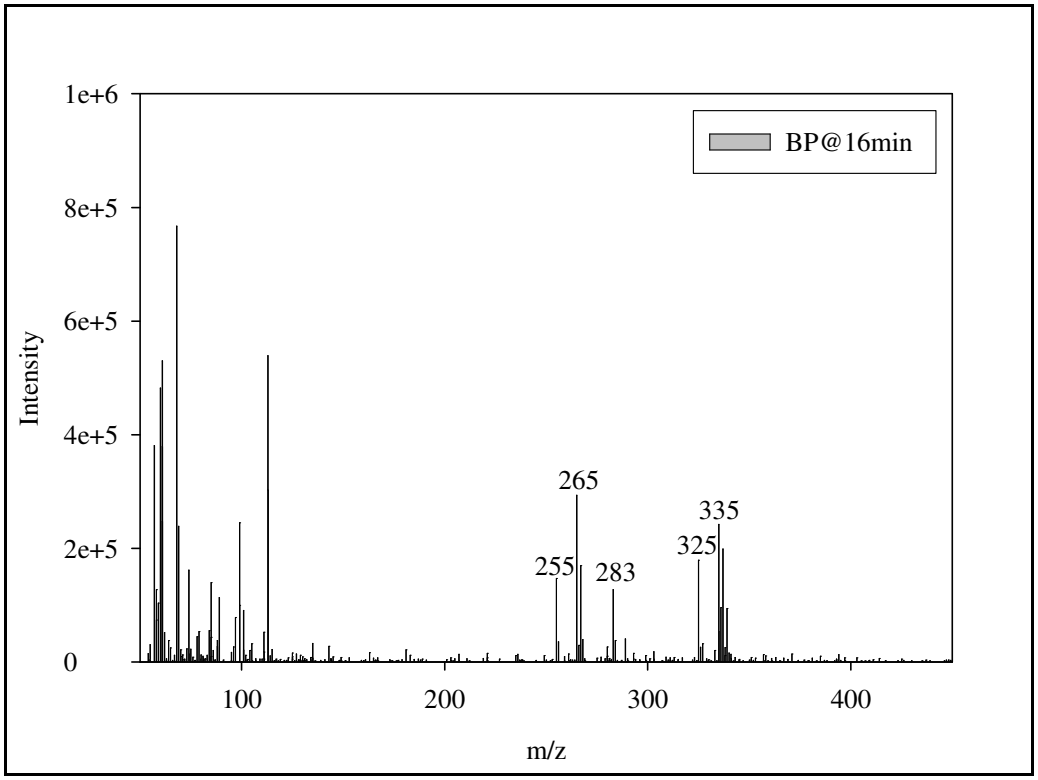


Figure B-17: Chlorination Byproduct of DES

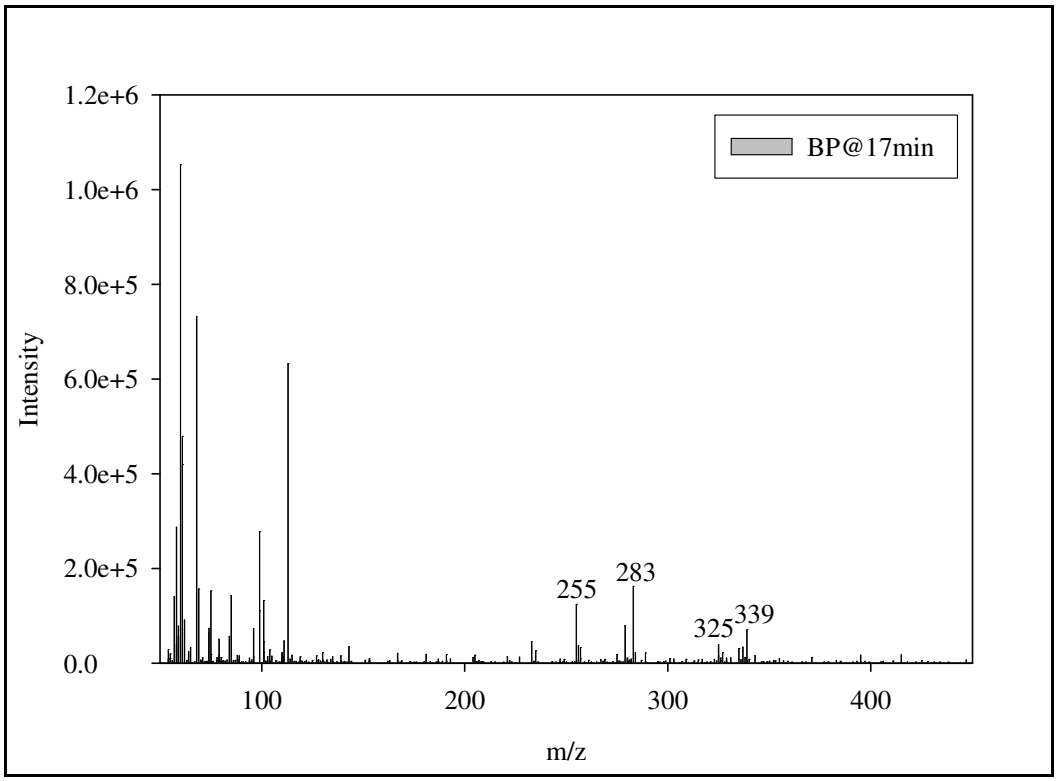


Figure B-18: Chlorination Byproduct of DES

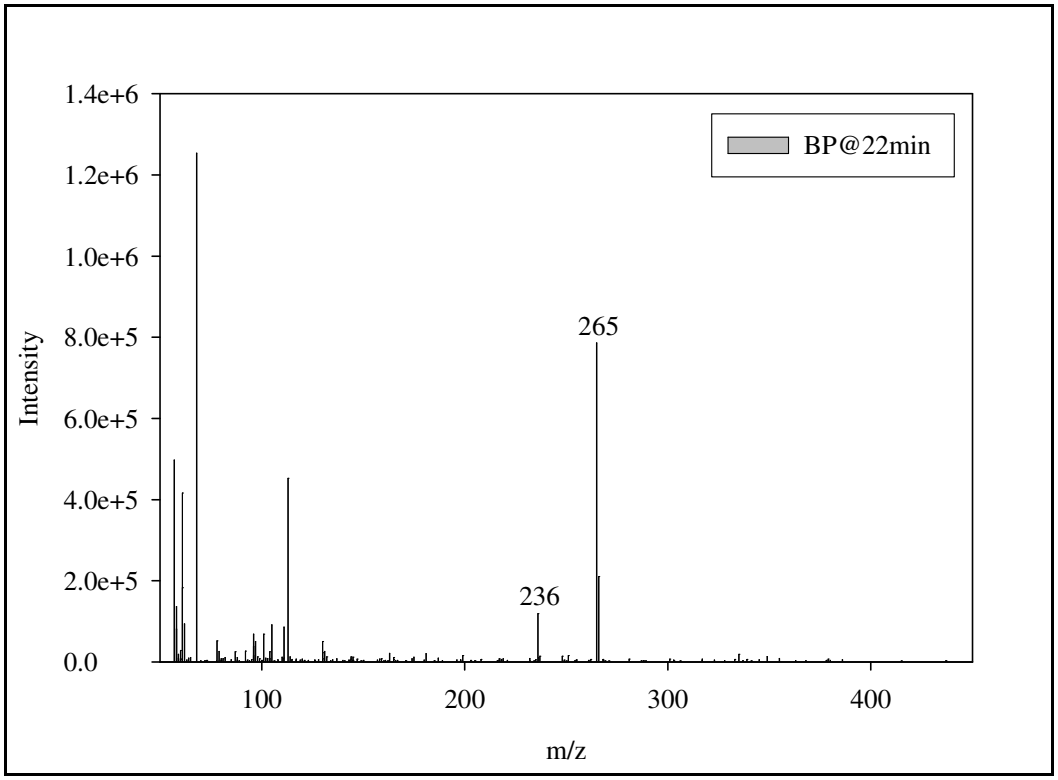


Figure B-19: Chlorination Byproduct of DES

EE2 and DES Chloramination Methods

EE2 and DES Chloramination (Pre-Ammoniation)
Initial ED concentration of 10 ± 0.1 uM
Total chlorine concentration of _____ uM
Total ammonia concentration of _____ uM
Chlorine/Nitrogen Ratio of 4 mg-Cl_2/mg-N
Temperature of solution 20 ºC

Sample times
0, 30, 60, 120, 300sec (5min), 600sec (10min), 1800sec (30min), 3600sec (1hr)

1) Sample size of 150 mL of 10 ± 0.1 uM EDC with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Inject _____mL of ammonia solution **then** _____mL of chlorine solution for final concentration of 35 uM and a Cl_2/N of 4. Mix solution thoroughly.

3) At time intervals of 0, 30, 60, 120, 300, 600, 1800, 3600sec, inject 100uL of sodium thiosulfate solution (100g/L) to quench the residual chloramines and stop the oxidation reaction. Measure pH after reaction.

4) Samples analyzed using HPLC to determine the remaining ED concentration.

EE2 and DES Chloramination (Simultaneous Addition)
Initial ED concentration of 10 ± 0.1 uM
Total chlorine concentration of _____ uM
Total ammonia concentration of _____ uM
Chlorine/Nitrogen Ratio of 4 mg-Cl_2/mg-N
Temperature of solution 20 ºC

Sample times
0, 30, 60, 120, 300sec (5min), 600sec (10min), 1800sec (30min), 3600sec (1hr)

1) Sample size of 150 mL of 10 ± 0.1 uM EDC with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Inject _____mL of ammonia solution and _____mL of chlorine solution **simultaneously** for final concentration of 35 uM and a Cl_2/N of 4. Mix solution thoroughly.

3) At time intervals of 0, 30, 60, 120, 300, 600, 1800, 3600sec, inject 100uL of sodium thiosulfate solution (100g/L) to quench the residual chloramines and stop the oxidation reaction. Measure pH after reaction.

4) Samples analyzed using HPLC to determine the remaining ED concentration.

EE2 and DES Chloramination (Pre-formed Chloramine Addition)
Initial ED concentration of 10 ± 0.1 uM
Total chlorine concentration of _____ uM
Total ammonia concentration of _____ uM
Chlorine/Nitrogen Ratio of 4 mg-Cl_2/mg-N
Temperature of solution 20 ºC

Sample times
0, 30, 60, 120, 300sec (5min), 600sec (10min), 1800sec (30min), 3600sec (1hr)

1) Sample size of 150 mL of 10 ± 0.1 uM EDC with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Make ammonia solution (0.7175 M) so that the final volume will be 100 mL and Cl_2/N of 4 when chlorine stock solution (35 µM) is added slowly to NH_2Cl solution. Stir for 10min. Measure residual. Inject _____mL of chloramine solution.

3) At time intervals of 0, 30, 60, 120, 300, 600, 1800, 3600sec, inject 100uL of sodium thiosulfate solution (100g/L) to quench the residual chloramines and stop the oxidation reaction. Measure pH after reaction.

4) Samples analyzed using HPLC to determine the remaining ED concentration.

EE2 and DES Chloramination (Pre-Chlorination Addition)
Initial ED concentration of 10 ± 0.1 uM
Total chlorine concentration of _____ uM
Total ammonia concentration of _____ uM
Chlorine/Nitrogen Ratio of 4 mg-Cl_2/mg-N
Temperature of solution 20 ºC

Sample times
0, 30, 60, 120, 300sec (5min), 600sec (10min), 1800sec (30min), 3600sec (1hr)

1) Sample size of 150 mL of 10 ± 0.1 uM EDC with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Inject _____mL chlorine of solution, wait 30 sec, **then** _____mL of ammonia solution for final concentration of 35 uM and a Cl_2/N of 4. Mix solution thoroughly.

3) At time intervals of 0, 30, 60, 120, 300, 600, 1800, 3600sec, inject 100uL of sodium thiosulfate solution (100g/L) to quench the residual chloramines and stop the oxidation reaction. Measure pH after reaction.

4) Samples analyzed using HPLC to determine the remaining ED concentration.

EE2 and DES Short and Long Chloramination (Pre-Chlorination Addition)
Initial ED concentration of 10 ± 0.1 uM
Total chlorine concentration of _____ uM
Total ammonia concentration of _____ uM
Chlorine/Nitrogen Ratio of 4 mg-Cl_2/mg-N
Temperature of solution 20 ºC

Sample times
5hrs, 48 hrs

1) Sample size of 150 mL of 10 ± 0.1 uM EDC with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Inject _____mL chlorine of solution, wait 30 sec, **then** _____mL of ammonia solution for final concentration of 1.5, 3.0 and 6.0 mg/L Cl_2 and a Cl_2/N of 4. Mix solution thoroughly.

3) After 5hrs and 48 hrs, measure free Cl_2, Mono, and Dichloramines. Inject 100uL of sodium thiosulfate solution (100g/L) to quench the residual chloramines and stop the oxidation reaction. Measure pH after reaction.

4) Samples analyzed using HPLC to determine the remaining ED concentration.

Table B-5: Pre-Ammoniation Chloramination of EE2

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
EE2	96.8	92.1	91.4	91.1	90.1	87.1	89.4	89.4
BP 1 (13min)	0	1.6	3.0	3.2	4.1	4.3	4.6	3.4
Other BPs	3.2	6.3	5.6	5.7	5.8	8.6	6.0	7.2

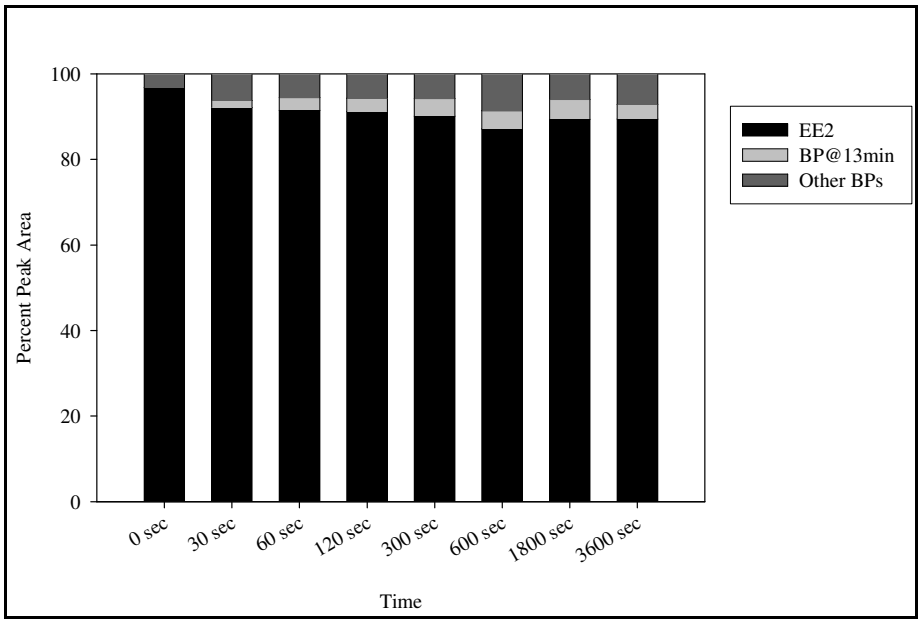


Figure B-20: Pre-Ammoniation Chloramination of EE2 Percent Peak Area

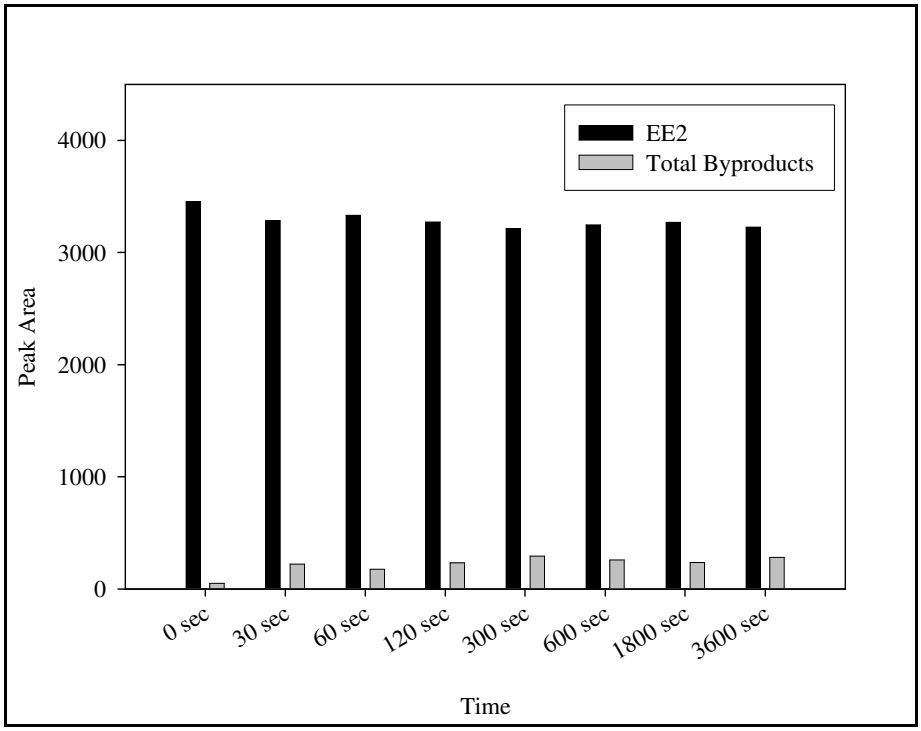


Figure B-21: Pre-Ammoniation Chloramination of EE2

Table B-6: Pre-Chlorination Chloramination of EE2

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
EE2	75.8	70.3	70.7	70.8	74.2	74.0	70.7	70.8
BP 1 (13min)	9.1	14.9	16.0	16.1	14.1	15.7	16.1	15.7
BP 2 (19min)	0	0.7	0.9	0.0	0.0	0.0	0.3	0.4
Other BPs	15.1	14.1	12.4	13.0	11.7	10.3	12.9	13.1

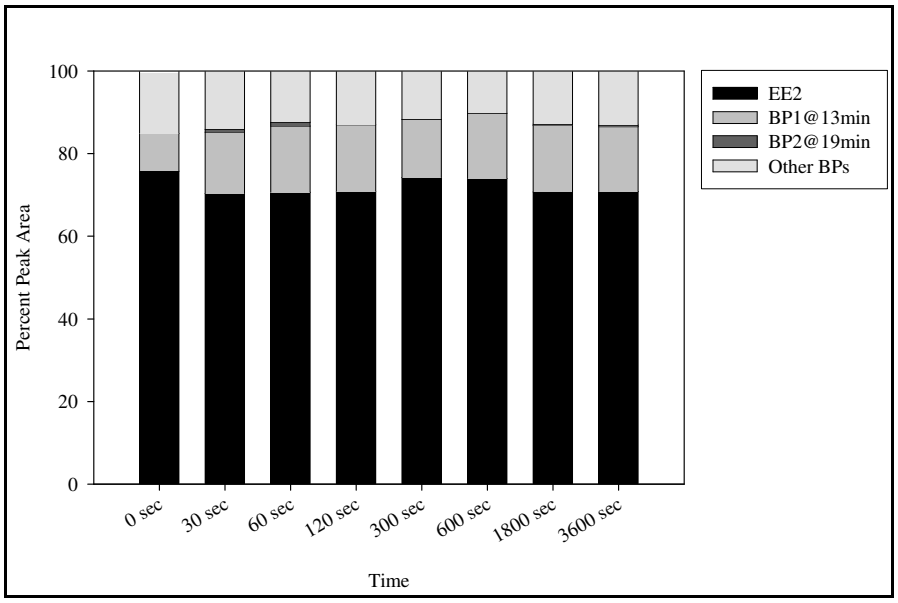


Figure B-22: Pre-Chlorination Chloramination of EE2 Percent Peak Area

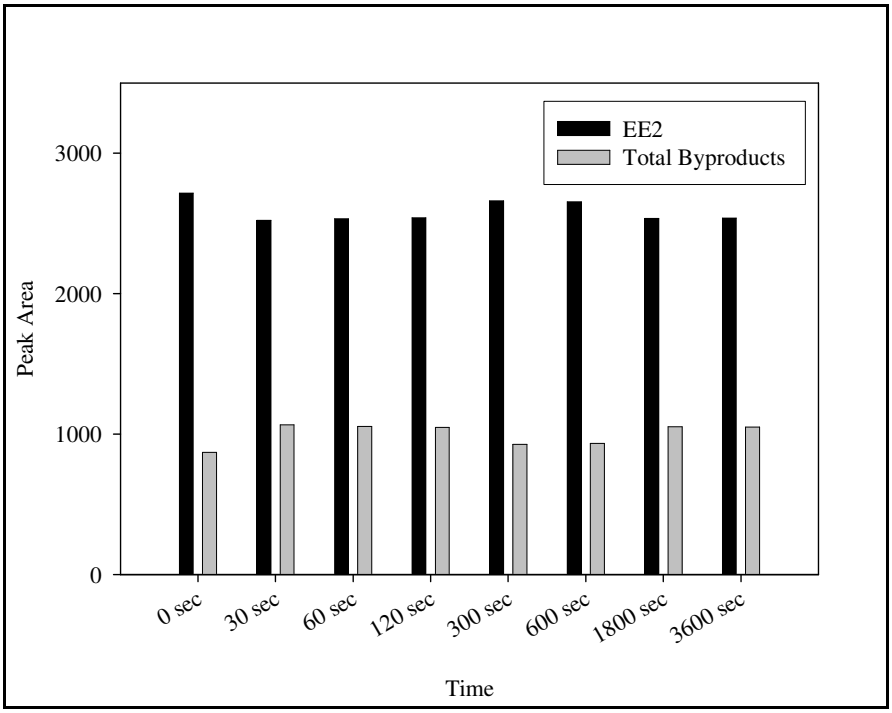


Figure B-23: Pre-Chlorination Chloramination of EE2

Table B-7: Simultaneous Addition Chloramination of EE2

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
EE2	93.5	90.3	87.9	95.3	95.9	93.4	92.0	91.2
BP 1 (13min)	0	3.0	3.9	4.4	2.7	2.2	3.8	3.8
BP 2 (19min)	0.6	0.6	1.2	0.3	0.0	0.1	0.5	1.5
Other BPs	5.9	6.0	7.0	0.0	1.4	4.3	3.8	3.4

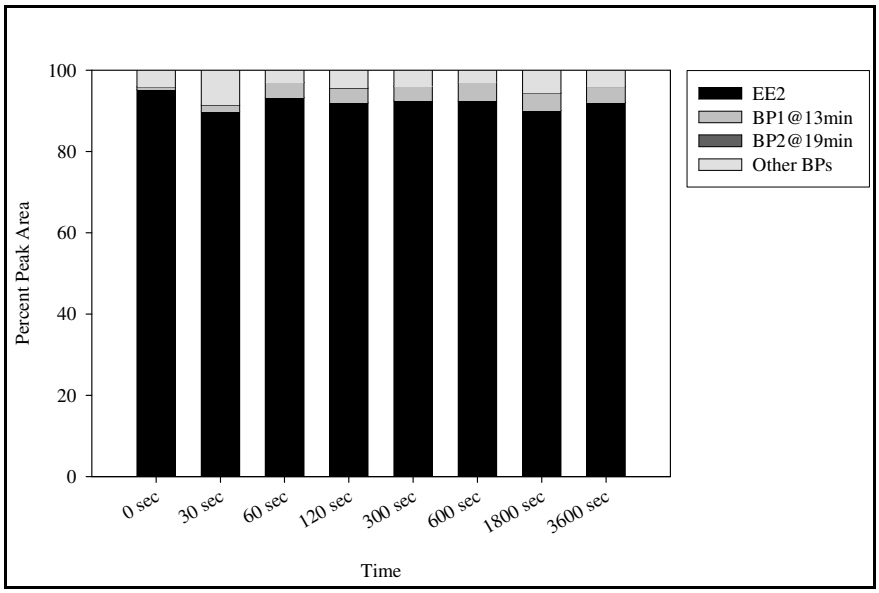


Figure B-24: Simultaneous Addition Chloramination of EE2 Percent Peak Area

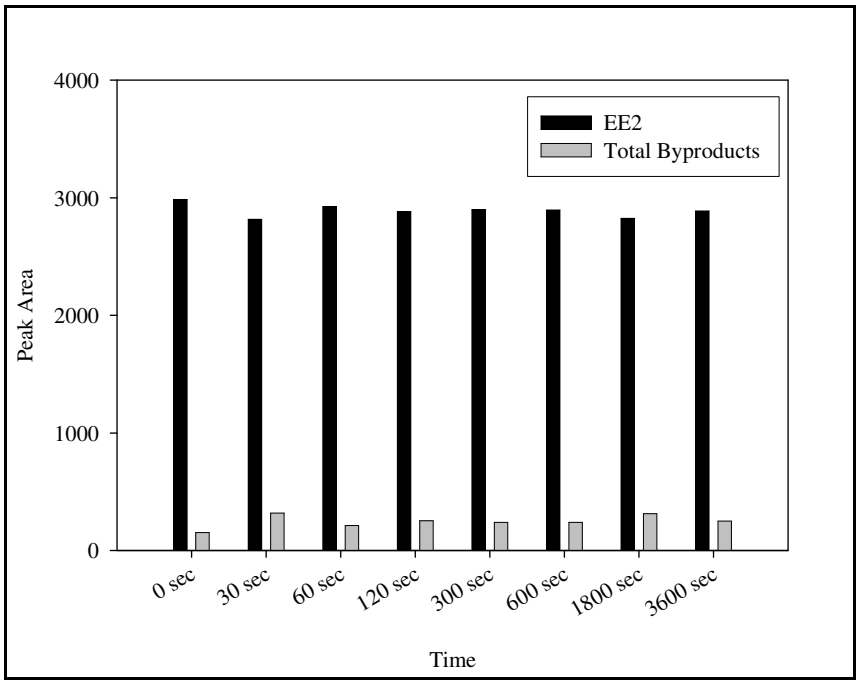


Figure B-25: Simultaneous Addition Chloramination of EE2

Table B-8: Pre-Formed Chloramine Chloramination of EE2

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
EE2	98.6	99.4	98.6	98.9	98.2	98.3	94.0	98.4
BP 1 (13min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 2 (19min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other BPs	1.4	0.6	1.4	1.1	1.8	1.7	6.0	1.6

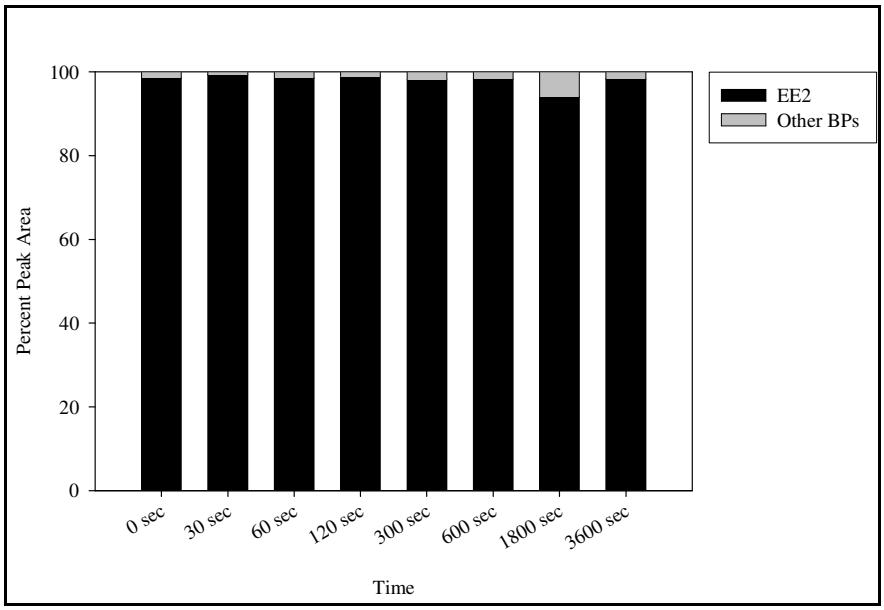


Figure B-26: Pre-Formed Chloramine Chloramination of EE2

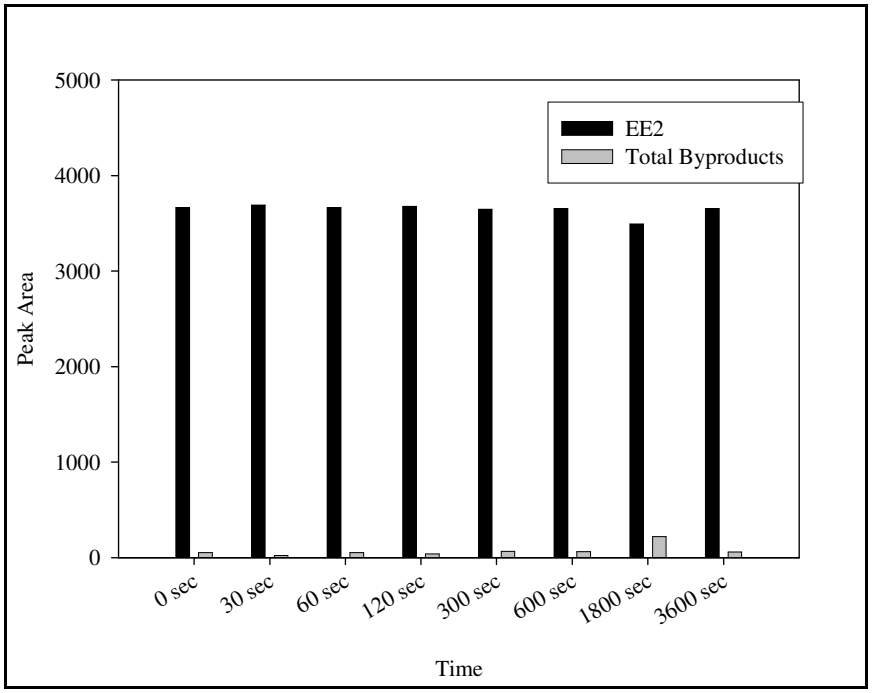


Figure B-27: Pre-Formed Chloramine Chloramination of EE2

Table B-9: Chloramination Residuals with Standard Deviations of EE2 Reactions

	Dose as Cl_2 (C:N of 4)	Cl_2 Residual (mg/L)	NH_2Cl Residual (mg/L)	$NHCl_2$ Residual (mg/L)	Concentration of EE2 (mg/L)
Pre-Ammoniation (3600 sec)	35 µM	0.00 ± 0	2.34 ± 0.02	0.04 ± 0.02	6.98 ± 0.15
Pre-Chlorination (3600 sec)	35 µM	0.00 ± 0	2.34 ± 0.08	0.00 ± 0	5.47 ±0.13
Simultaneous Addition (3600 sec)	35 µM	0.05 ± 0.05	2.36 ± 0.05	0.20 ± 0.23	5.86 ± 0.08
Pre-Mixed Chloramination (3600 sec)	35 µM	0.00 ± 0	1.78 ± 0.07	0.06 ± 0.01	7.20 ±0.11
Short Term Pre-Chlorination	1.5 mg/L	0.00 ± 0	1.26 ±0.05	0.00 ± 0	5.78 ± 0.03
	3.0 mg/L	0.00 ± 0	2.33 ± 0.21	0.28 ± 0.50	4.64 ± 0.16
	6.0 mg/L	0.07 ± 0.04	5.11 ± 0.15	0.02 ± 0.05	3.41 ± 0.03
Long Term Pre-Chlorination	1.5 mg/L	0.00 ± 0	1.05 ± 0.03	0.01 ± 0.01	5.32 ± 0.03
	3.0 mg/L	0.00 ± 0	2.35 ± 0.43	0.05 ± 0.05	3.65 ± 0.33
	6.0 mg/L	0.00 ± 0	4.15 ± 0.13	0.07 ± 0.06	2.70 ± 0.04

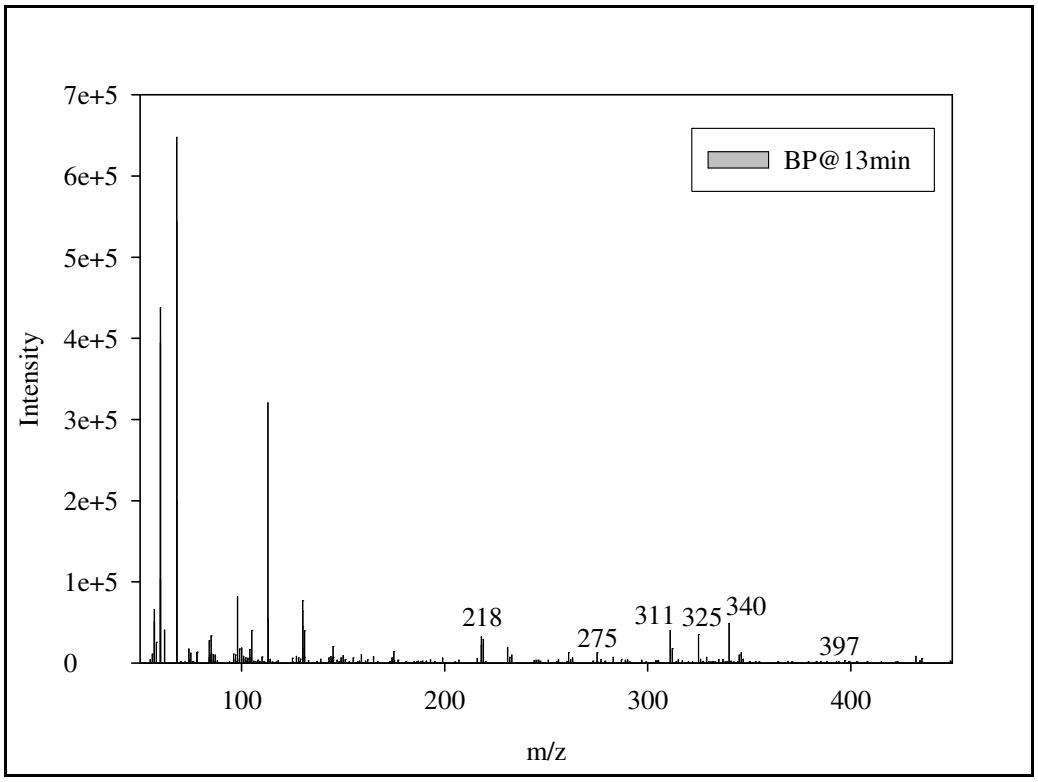


Figure B-28: Chloramination Byproduct of EE2

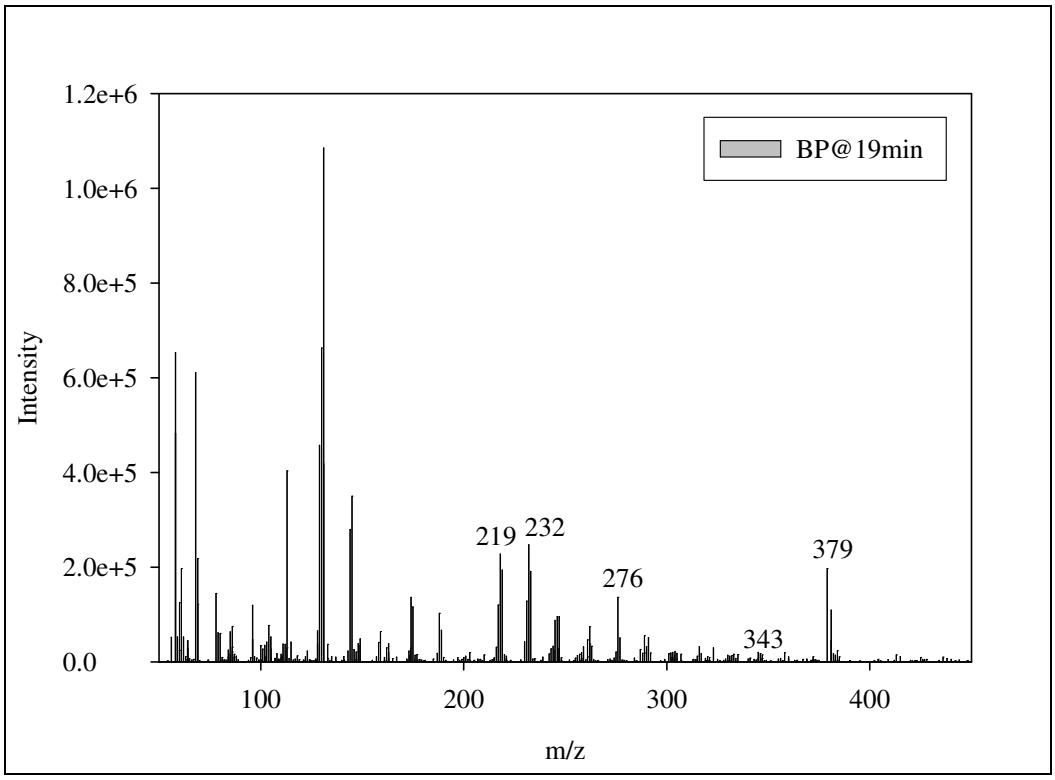


Figure B-29: Chloramination Byproduct of EE2

Table B-10: Pre-Ammoniation Chloramination of DES

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
DES	99.5	98.2	96.2	94.7	95.9	94.4	94.9	93.3
BP 1 (13min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 2 (15min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 3 (17min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other BPs	0.5	1.8	3.8	5.3	4.1	5.6	5.1	6.7

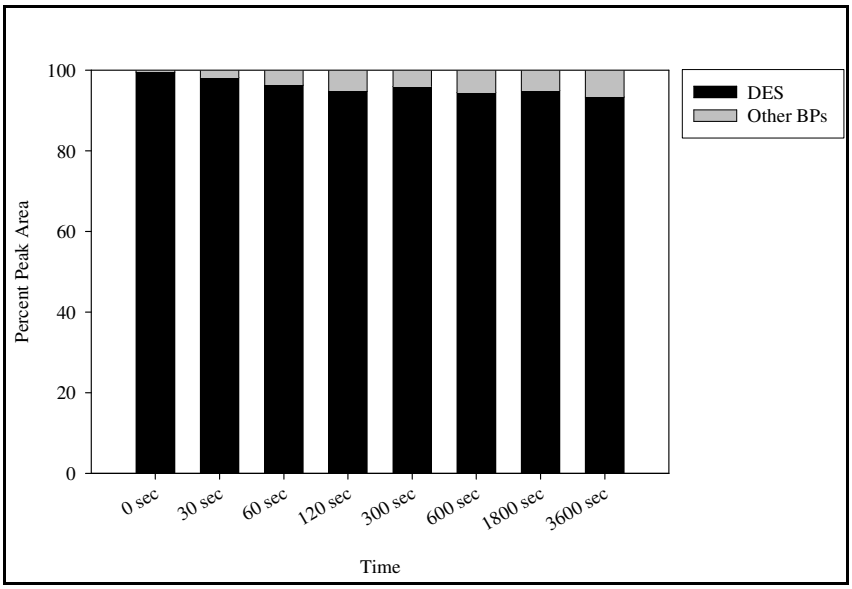


Figure B-30: Pre-Ammoniation Chloramination of DES Percent Peak Area

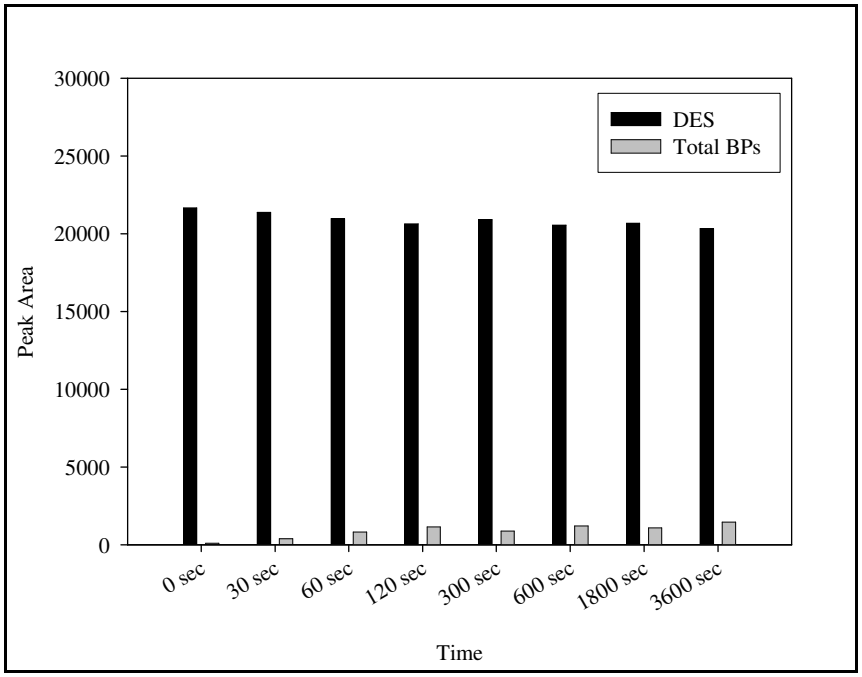


Figure B-31: Pre-Ammoniation Chloramination of DES

Table B-11: Pre-Chlorination Chloramination of DES

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
DES	87.7	82.2	82.5	84.4	84.3	83.1	82.6	83.5
BP 1 (13min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 2 (15min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 3 (17min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other BPs	12.3	17.8	17.5	15.6	15.7	16.9	17.4	16.5

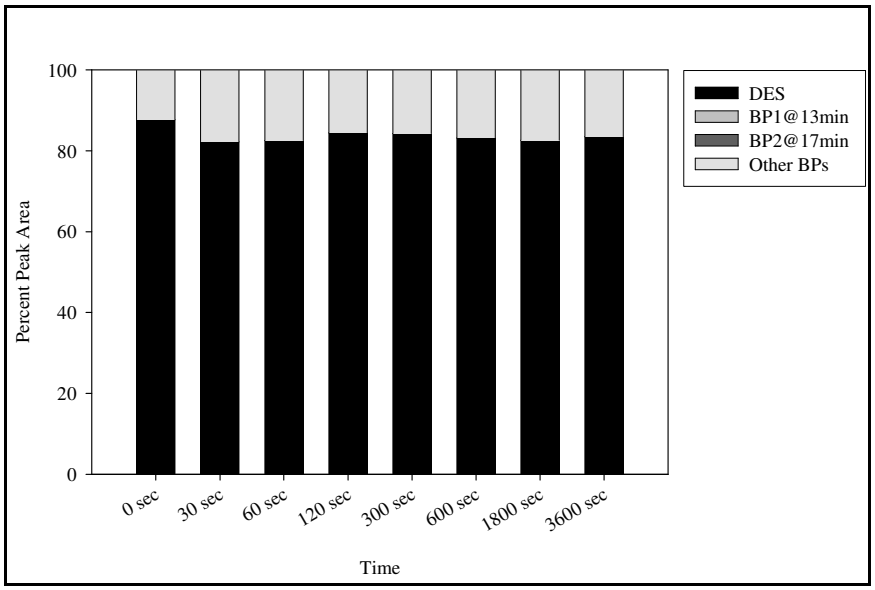


Figure B-32: Pre-Chlorination Chloramination of DES Percent Peak Area

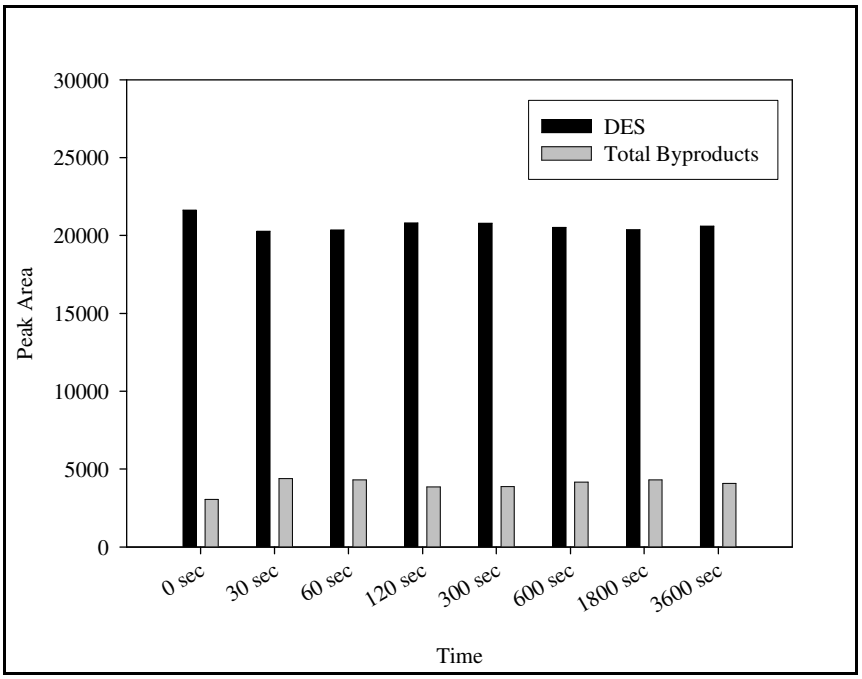


Figure B-33: Pre-Chlorination Chloramination of DES

Table B-12: Simultaneous Addition Chloramination of DES

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
DES	94.6	93.9	95.2	94.8	92.8	94.9	93.5	92.2
BP 1 (13min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 2 (15min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 3 (17min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other BPs	5.4	6.1	4.8	5.2	7.2	5.1	6.5	7.8

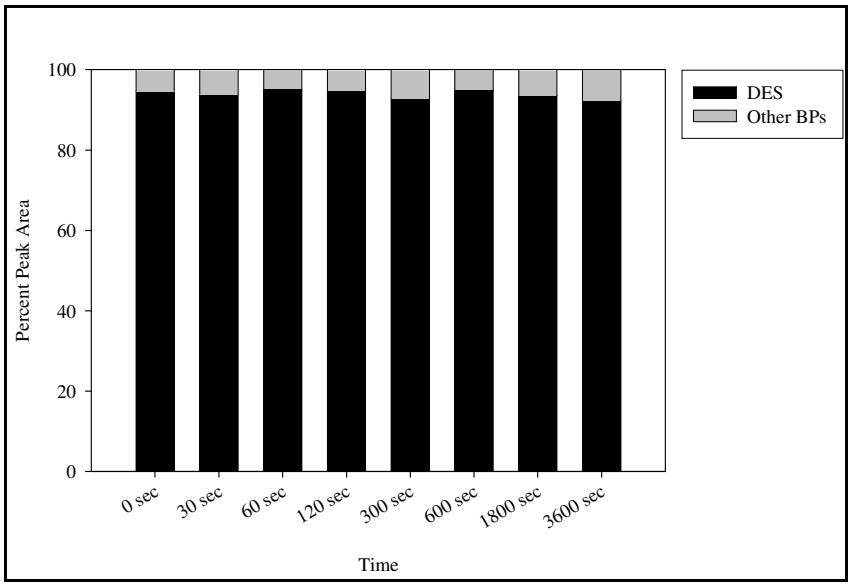


Figure B-34: Simultaneous Addition Chloramination of DES Percent Peak Area

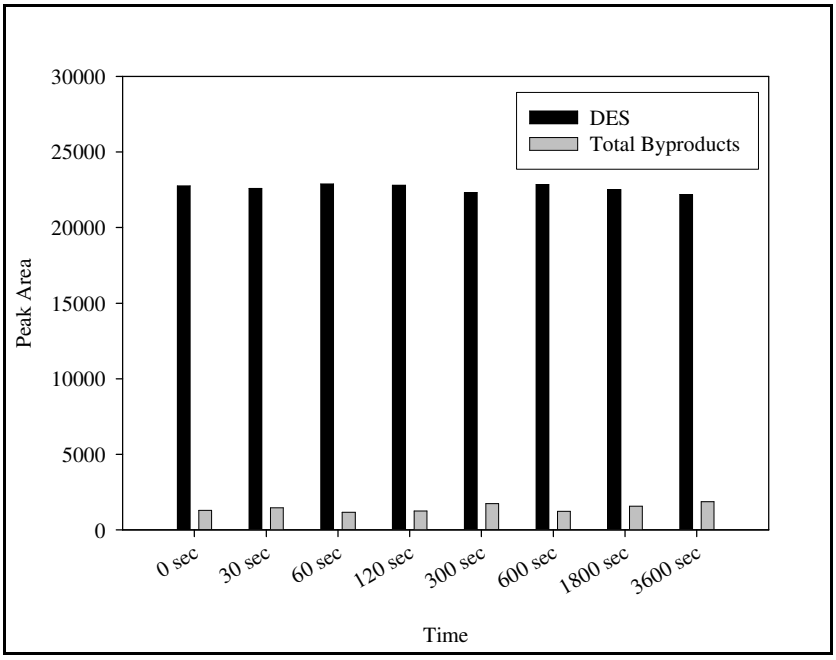


Figure B-35: Simultaneous Addition Chloramination of DES

Table B-13: Pre-Formed Chloramine Chloramination of DES

	% Total Peak Areas							
	0 sec	**30 sec**	**60 sec**	**120 sec**	**300 sec**	**600 sec**	**1800 sec**	**3600 sec**
DES	97.0	93.8	94.0	97.8	100.0	98.3	100.1	99.2
BP 1 (13min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 2 (15min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BP 3 (17min)	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other BPs	3.0	6.2	6.0	2.2	0.0	1.7	-0.1	0.8

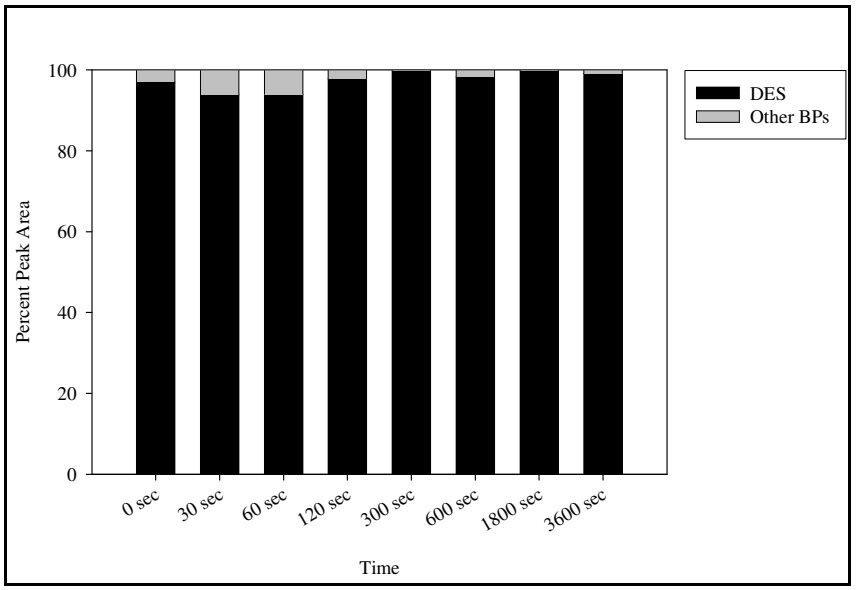


Figure B-36: Pre-Formed Chloramine Chloramination of DES Percent Peak Area

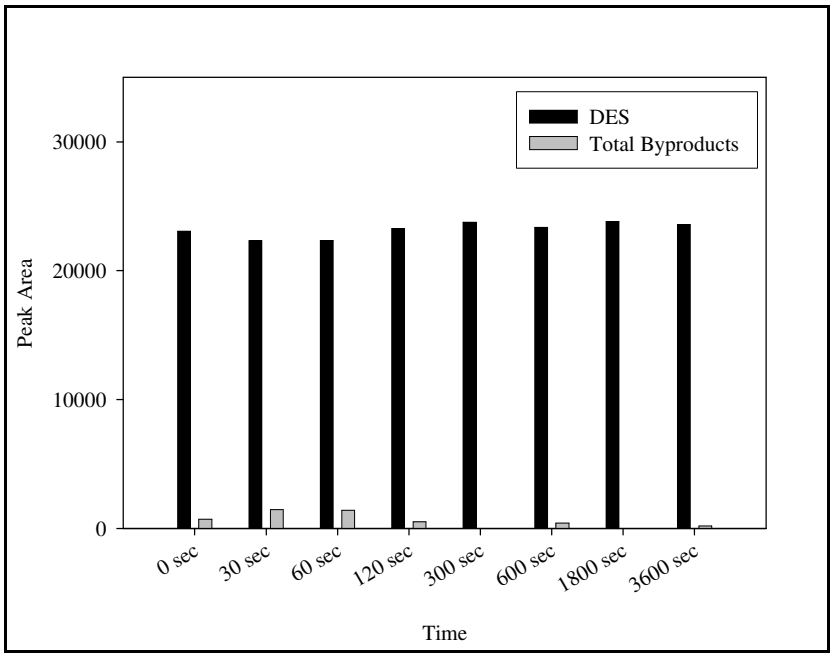


Figure B-37: Pre-Formed Chloramine Chloramination of DES

Table B-14: Chloramination Residuals with Standard Deviations of DES Reactions

	Dose as Cl_2 (C:N of 4)	Cl_2 Residual (mg/L)	NH_2Cl Residual (mg/L)	$NHCl_2$ Residual (mg/L)	Concentration of DES (mg/L)
Pre-Ammoniation (3600 sec)	35 µM	0.00 ± 0	1.98 ± 0.57	0.09 ± 0.03	26.32 ± 0.81
Pre-Chlorination (3600 sec)	35 µM	0.00 ± 0	2.43 ± 0.04	0.00 ± 0	27.07 ± 0.52
Simultaneous Addition (3600 sec)	35 µM	0.01 ± 0.01	2.33 ± 0.19	0.05 ± 0.02	28.84 ± 0.42
Pre-Mixed Chloramination (3600 sec)	35 µM	0.00 ± 0	1.69 ±0.11	0.08 ± 0.02	32.57 ± 2.17
Short Term Pre-Chlorination	1.5 mg/L	0.00 ± 0	1.27 ± 0.03	0.01 ± 0.01	28.54 ± 0.05
	3.0 mg/L	0.00 ± 0	2.60 ± 0.05	0.03 ± 0.01	25.68 ± 0.12
	6.0 mg/L	0.03 ± 0.02	5.46 ± 0.10	0.03 ± 0.06	19.45 ± 0.20
Long Term Pre-Chlorination	1.5 mg/L	0.00 ± 0	1.04 ± 0.02	0.01 ± 0.02	34.53 ± 0.44
	3.0 mg/L	0.00 ± 0	2.22 ± 0.04	0.00 ± 0	28.93 ± 0.58
	6.0 mg/L	0.00 ± 0	4.02 ± 0.08	0.04 ± 0.04	19.60 ± 0.35

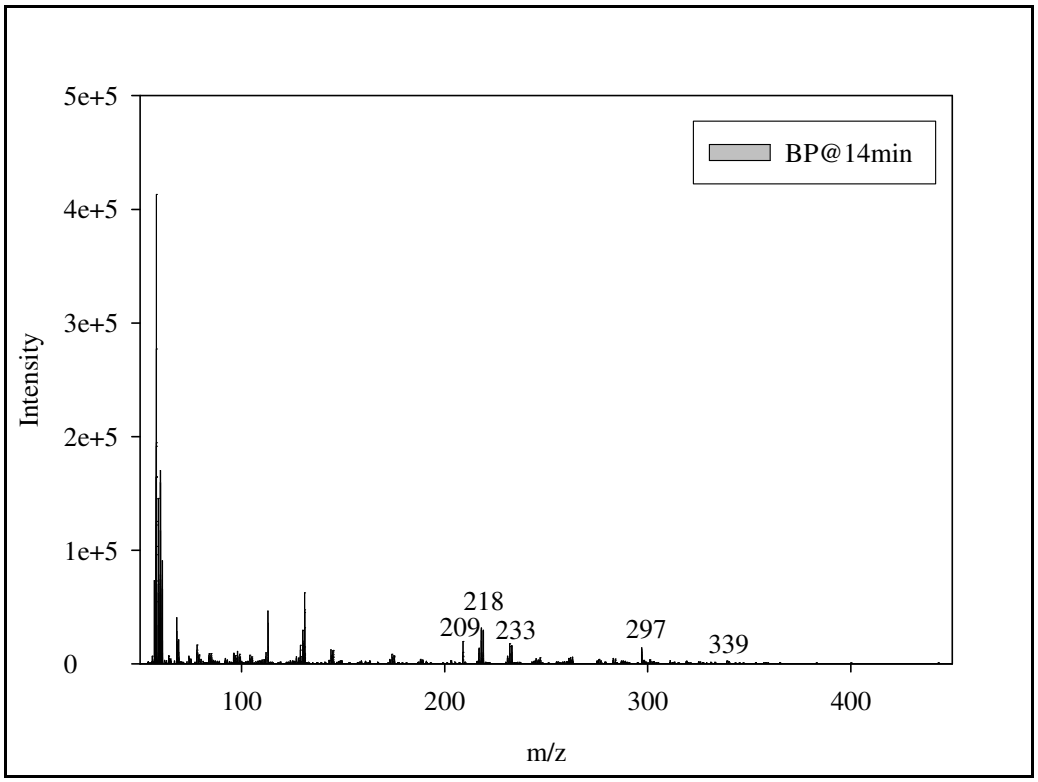


Figure B-38: Chloramination Byproducts of DES

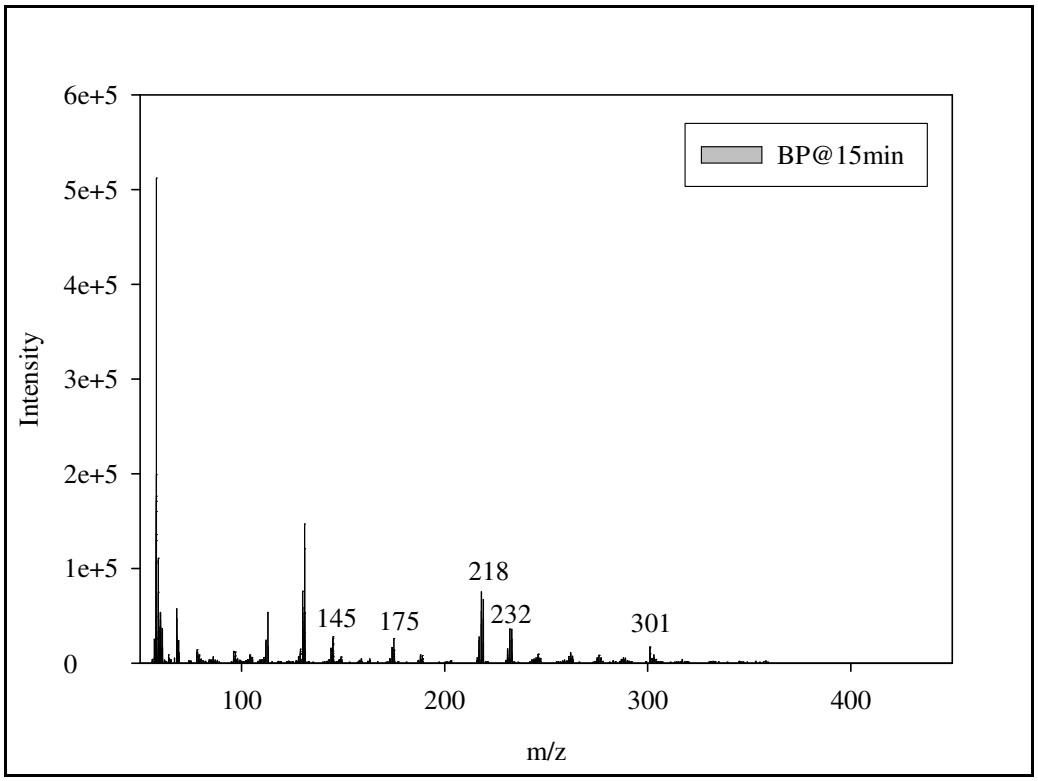


Figure B-39: Chloramination Byproducts of DES

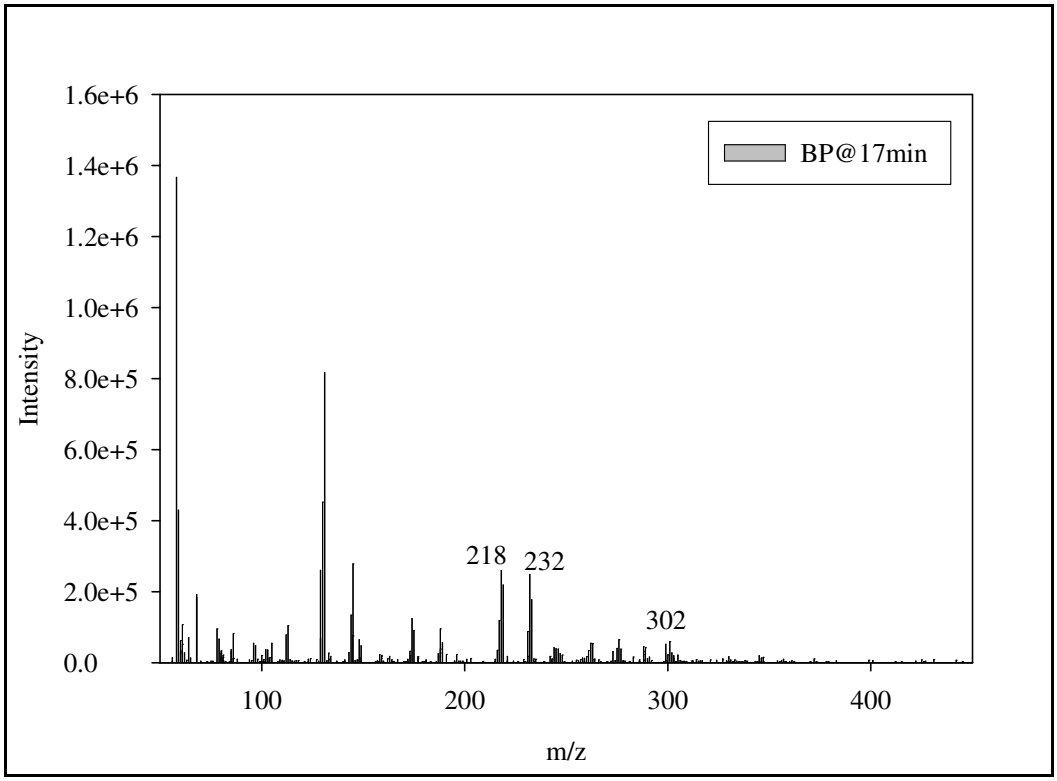


Figure B-40: Chloramination Byproducts of DES

Ozone Treatment of EDCs

EDC Instantaneous Ozonation

Initial ED concentration of 10 ± 0.1 uM
Temperature of solution 20 ºC

Ozone Doses
1, 2, 4, 6, 8, 10 uM

1) Sample size of 50 mL of 10 ± 0.1 uM EDC with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Bubble oxygen through 10^{-4}M HCl solution to produce O_3. Measure concentration at 258 nm to determine dose amounts ($\varepsilon_{258\ nm} = 3000\ M^{-1}cm^{-1}$).

3) Inject ozone solution for final concentrations of 1, 2, 4, 6, 8, 10 uM. Mix solution thoroughly. Allow reaction to run to completion.

4) Measure pH after reaction. Measure O_3 absorbance after dosing.

5) Samples analyzed using HPLC to determine the remaining ED concentration.

EE2 Short Term Ozonation

Initial ED concentration of 10 ± 0.1 uM
Temperature of solution 20 ºC

Ozone Doses
1.5, 2.0, 3.0, 6.0 mg/L

1) Sample size of 50 mL of 10 ± 0.1 uM EE2 with a pH adjustment to 7.0 ± 0.5 with 10 mM phosphoric acid buffer.

2) Bubble oxygen through 10^{-4}M HCl solution to produce O_3. Measure concentration at 258 nm to determine dose amounts ($\varepsilon_{258\ nm} = 3000\ M^{-1}cm^{-1}$).

3) Inject ozone solution for final concentrations of 1.5, 2.0, 3.0, 6.0 mg/L. Mix solution thoroughly. Allow reaction to run to completion.

4) Measure pH after reaction. Measure O_3 absorbance after dosing.

5) Samples analyzed using HPLC to determine the remaining ED concentration.

Table B-15: Instantaneous Ozonation of EE2 (ESI Negative)

	% Total Peak Areas					
	1 uM	**2 uM**	**4 uM**	**6 uM**	**8 uM**	**10 uM**
EE2	97.9	96.4	89.7	85.7	80.5	76.6
Byproduct 1 (9min)	0.0	0.0	0.0	0.0	0.0	0.0
Byproduct 2 (11min)	0.0	0.2	0.9	1.2	1.6	2.0
Byproduct 3 (13min)	0.0	0.7	1.4	3.5	5.0	6.6
Byproduct 4 (15min)	0.0	0.0	0.0	0.0	0.0	0.0
Byproduct 5 (19min)	0.0	0.0	0.0	0.0	0.0	0.0
Other BPs	2.1	2.7	8.0	9.6	13.0	14.8

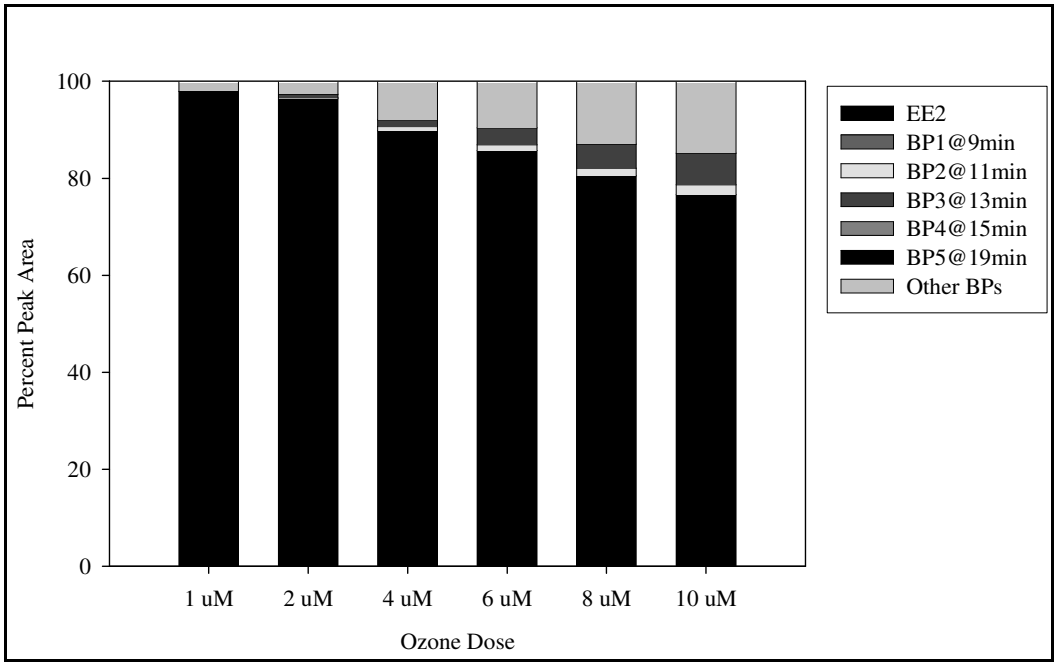


Figure B-41: Ozonation of EE2 Percent Peak Area (ESI Negative)

Table B-16: Instantaneous Ozonation of EE2 (ESI Positive)

	% Total Peak Areas					
	1 uM	**2 uM**	**4 uM**	**6 uM**	**8 uM**	**10 uM**
EE2	90.6	86.9	84.7	78.1	75.1	67.7
Byproduct 9 (10min)	0.0	0.0	0.9	1.3	1.9	2.4
Byproduct 14 (16min)	0.0	0.0	0.0	0.0	0.4	0.5
Other Byproducts	9.4	13.1	14.4	20.6	22.6	29.4

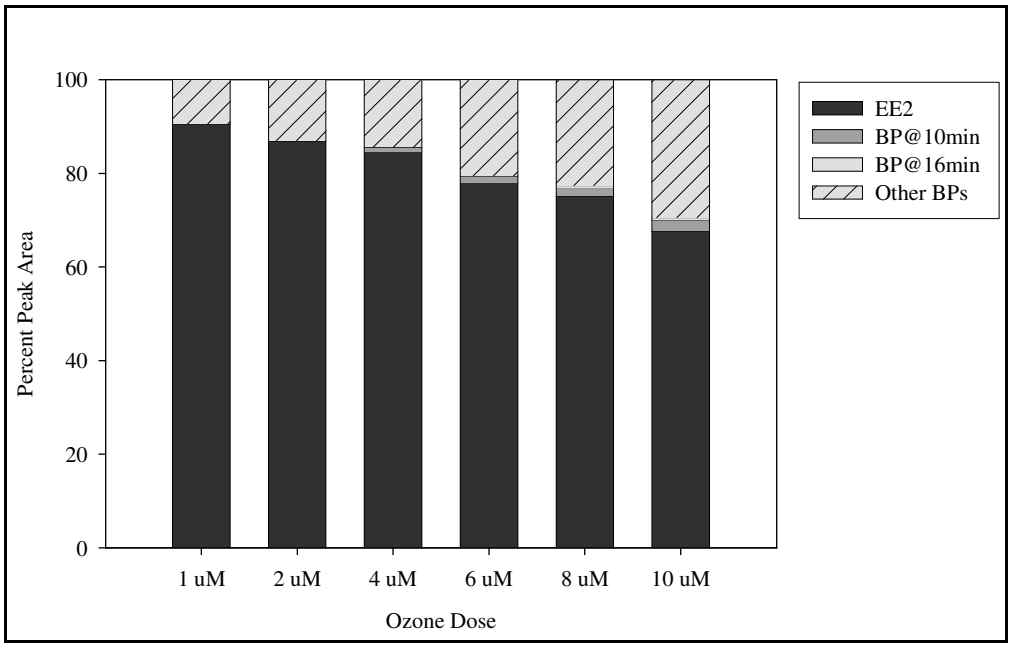


Figure B-42: Ozonation of EE2 Percent Peak Area (ESI Positive)

Table B-17: Short Term Ozonation of EE2 (ESI Negative)

	% Total Peak Areas			
	1.5 mg/L	**2.0 mg/L**	**3.0 mg/L**	**6.0 mg/L**
EE2	56.3	43.4	26.4	2.5
Byproduct 1 (9min)	0.6	0.9	1.5	3.3
Byproduct 2 (11min)	6.4	8.8	12.3	19.9
Byproduct 3 (13min)	8.5	14.2	14.7	8.1
Byproduct 4 (15min)	0.0	0.0	0.0	1.4
Byproduct 5 (19min)	0.5	1.3	3.1	13.4
Other Byproducts	27.7	31.3	42.0	51.5

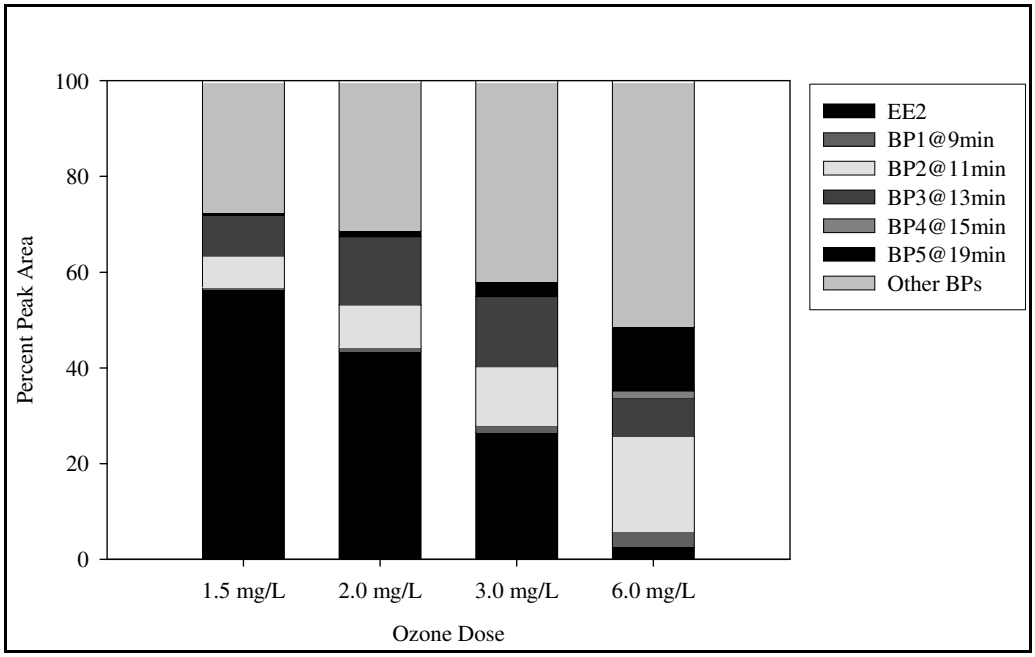


Figure B-43: Short Term Ozonation of EE2 Percent Peak Area (ESI Negative)

Table B-18: Short Term Ozonation of EE2 (ESI Positive)

	% Total Peak Areas			
	1.5 mg/L	**2.0 mg/L**	**3.0 mg/L**	**6.0 mg/L**
EE2	42.4	32.9	16.1	0.0
Byproduct 6 (7min)	0.6	0.5	0.6	0.4
Byproduct 7 (8min)	0.5	0.3	0.1	0.4
Byproduct 8 (9min)	1.2	1.3	5.6	2.0
Byproduct 9 (10min)	8.1	8.7	14.0	21.1
Byproduct 10 (12min)	6.2	6.0	4.9	2.8
Byproduct 11 (13min)	0.0	0.2	0.3	0.6
Byproduct 12 (14min)	0.0	0.0	0.0	0.2
Byproduct 13 (15min)	0.7	0.4	0.4	0.7
Byproduct 14 (16min)	1.6	1.7	2.1	1.4
Byproduct 15 (17min)	0.5	0.3	0.4	0.7
Byproduct 16 (19min)	0.9	1.7	4.9	10.3
Other Byproducts	37.4	46.0	50.5	59.3

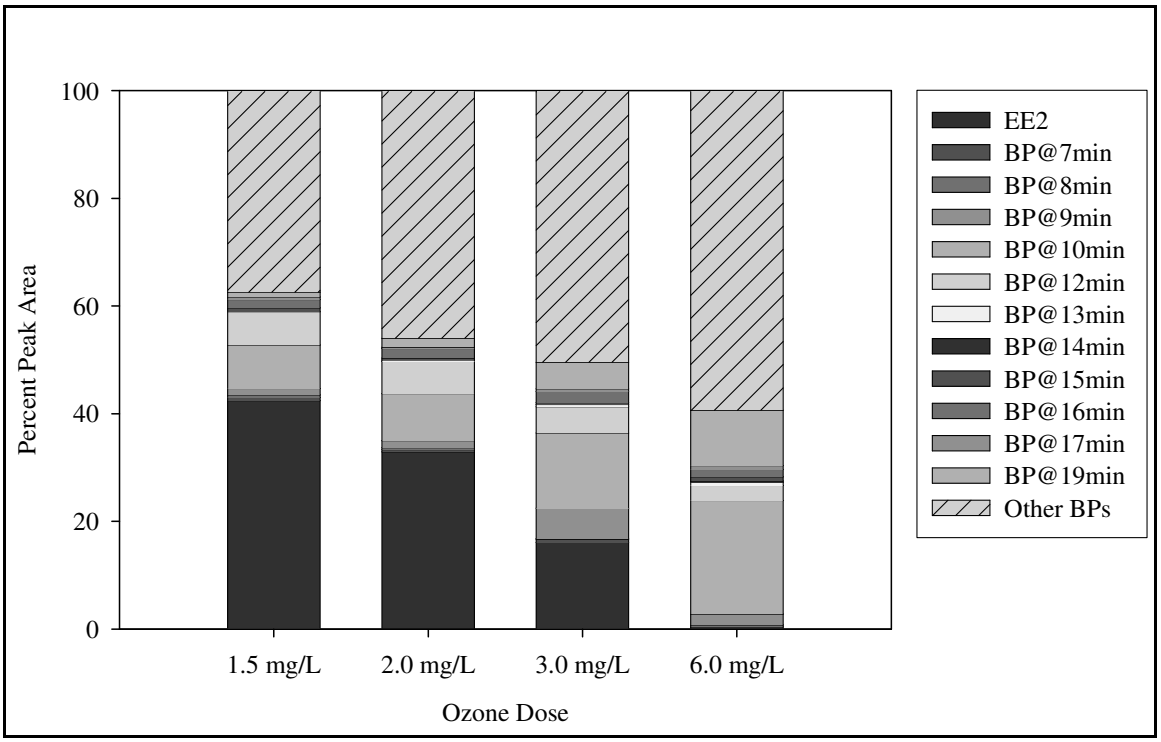


Figure B-44: Short Term Ozonation of EE2 Percent Peak Area (ESI Positive)

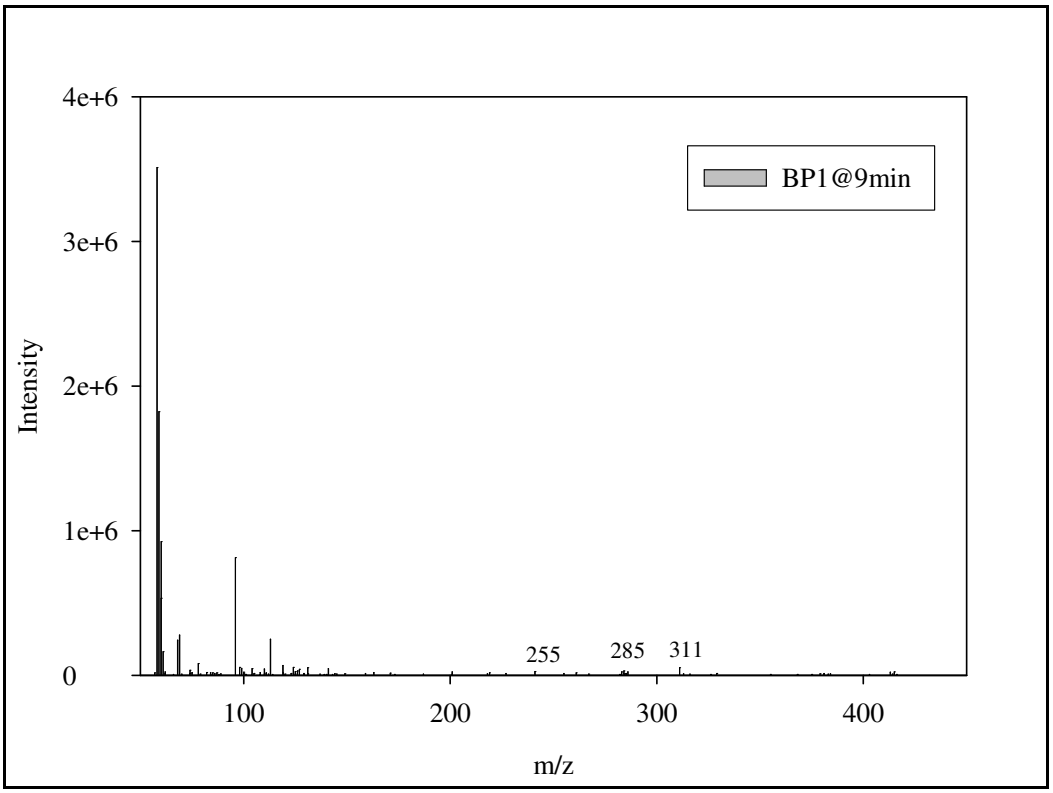


Figure B-45: Ozonation Byproducts of EE2 (ESI Negative)

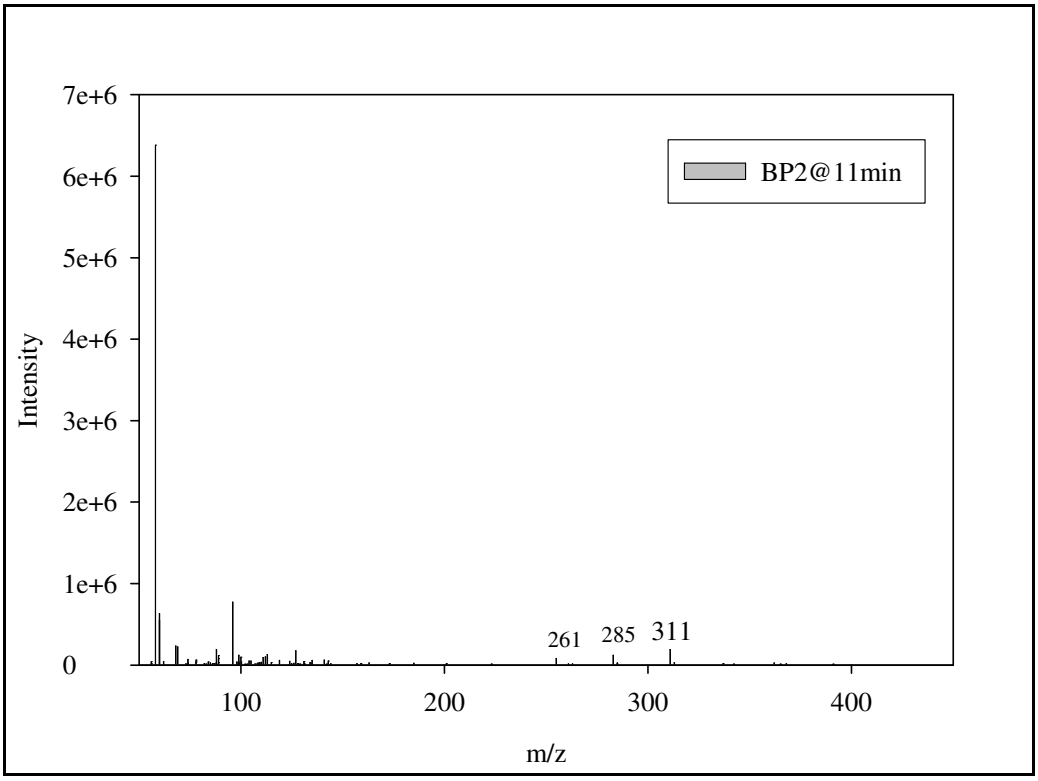


Figure B-46: Ozonation Byproducts of EE2 (ESI Negative)

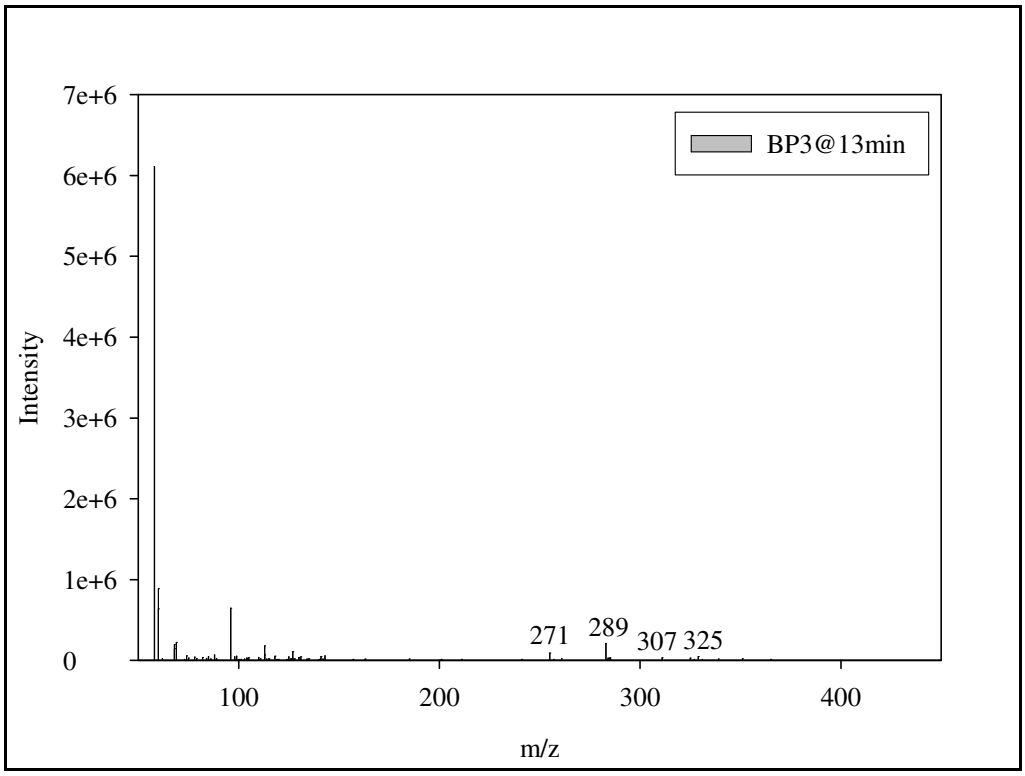


Figure B-47: Ozonation Byproducts of EE2 (ESI Negative)

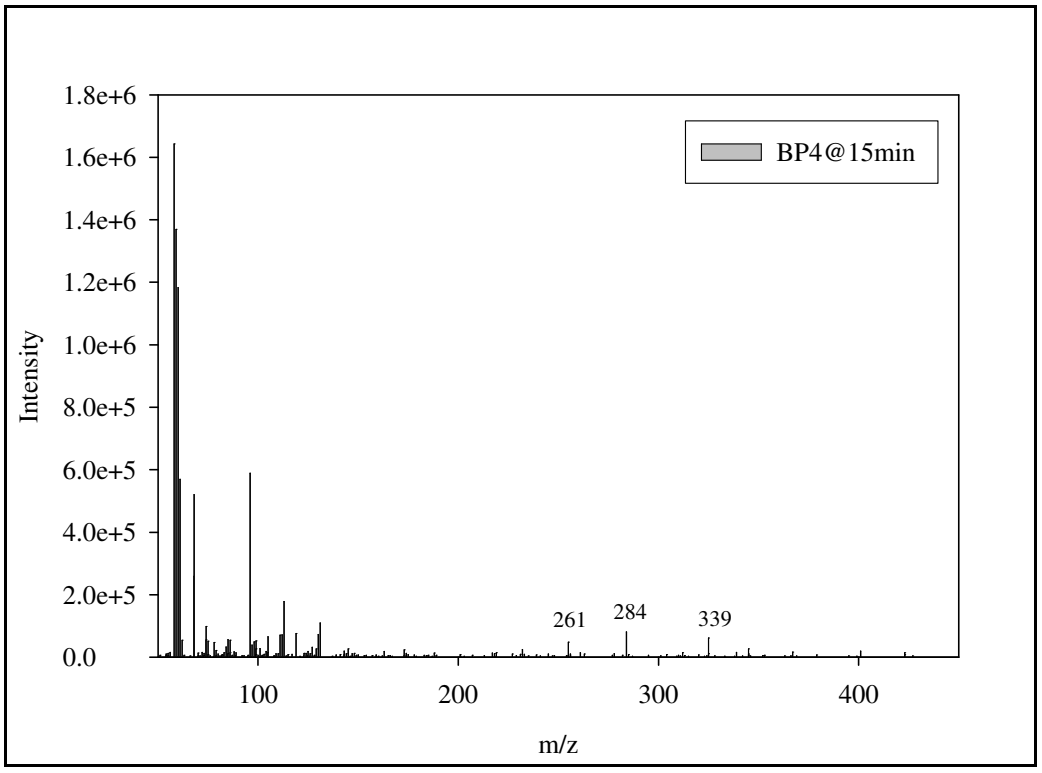


Figure B-48: Ozonation Byproducts of EE2 (ESI Negative)

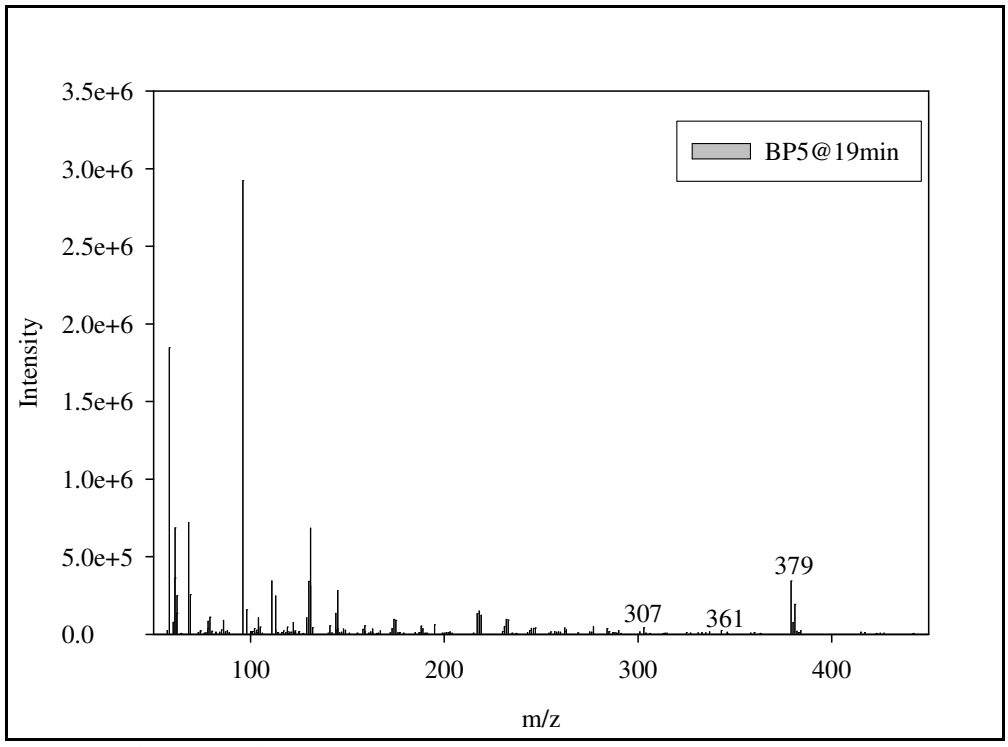


Figure B-49: Ozonation Byproducts of EE2 (ESI Negative)

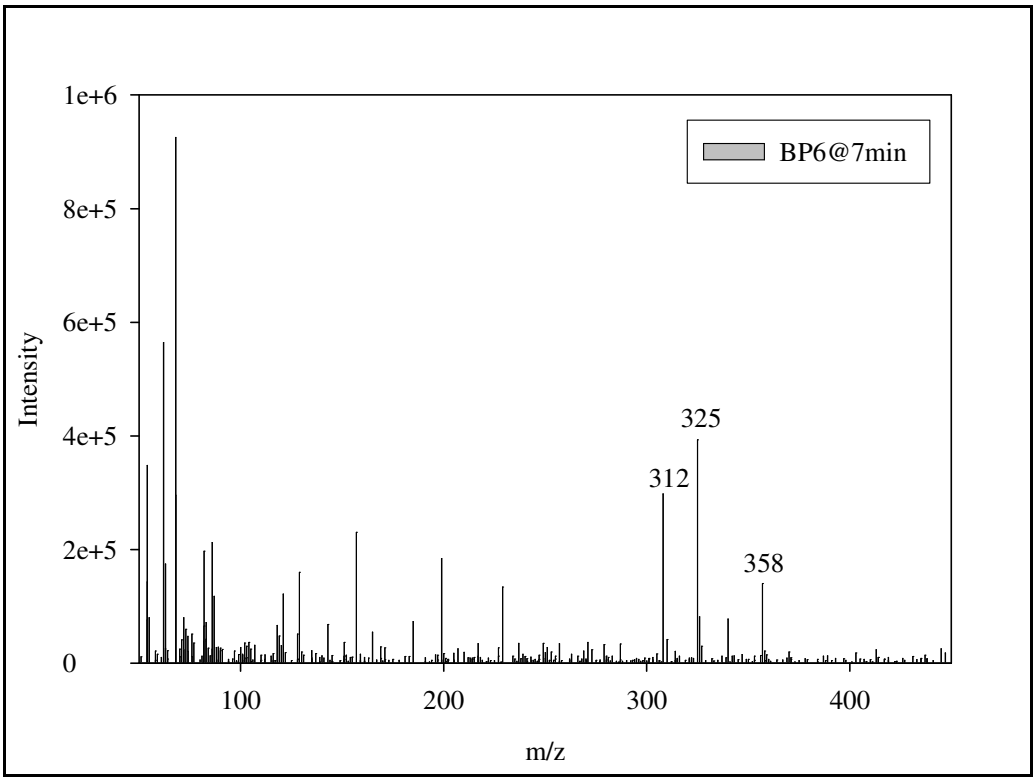


Figure B-50: Ozonation Byproducts of EE2 (ESI Positive)

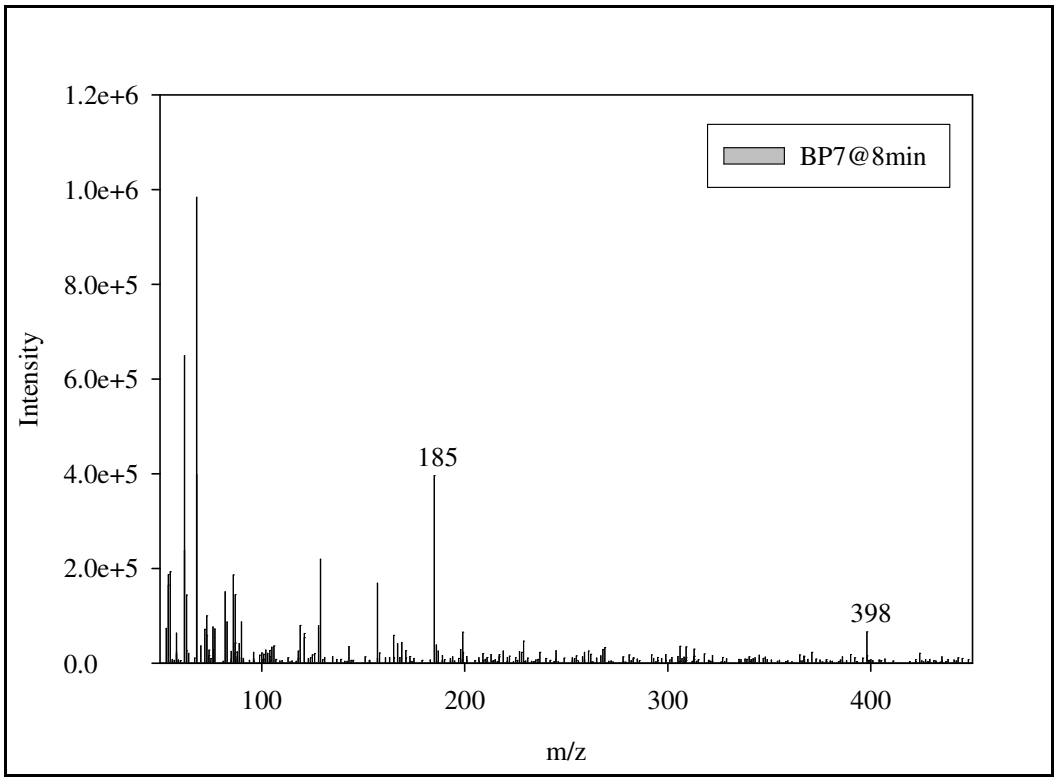


Figure B-51: Ozonation Byproducts of EE2 (ESI Positive)

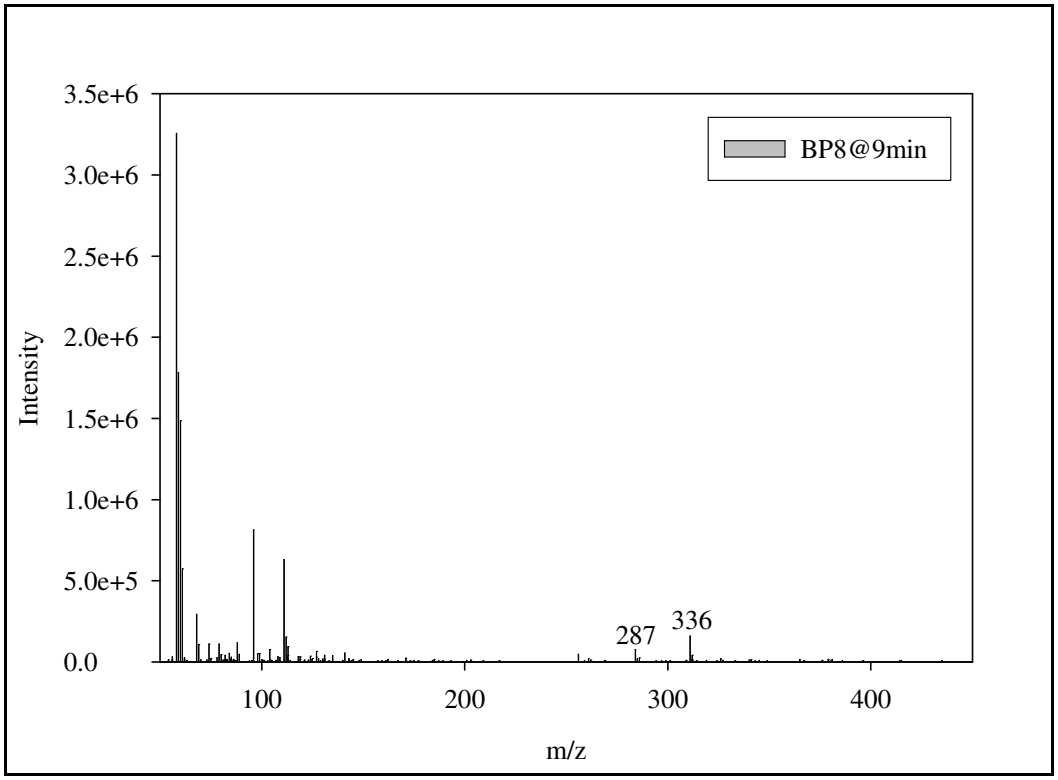


Figure B-52: Ozonation Byproducts of EE2 (ESI Positive)

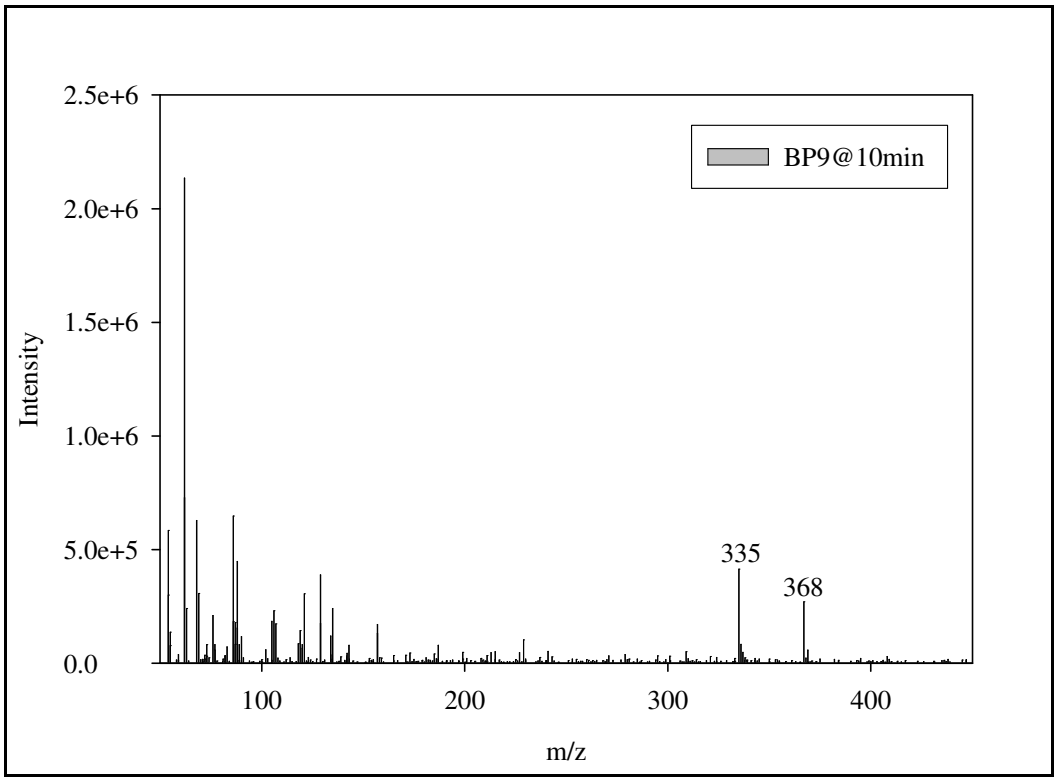


Figure B-53: Ozonation Byproducts of EE2 (ESI Positive)

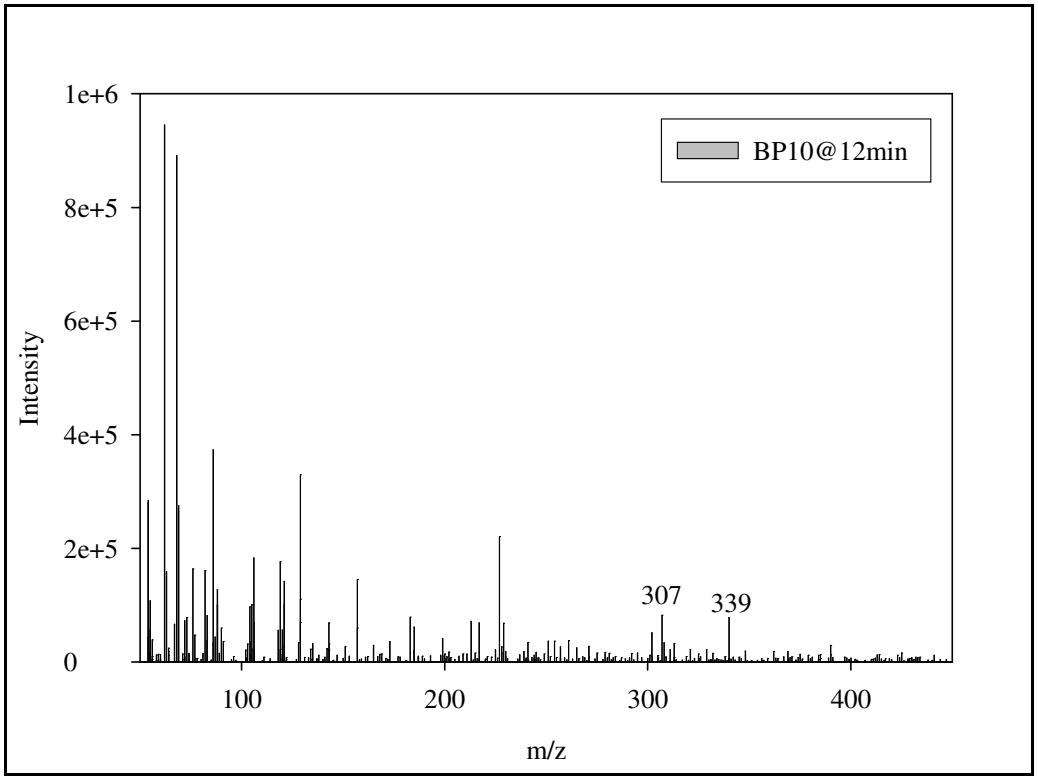


Figure B-54: Ozonation Byproducts of EE2 (ESI Positive)

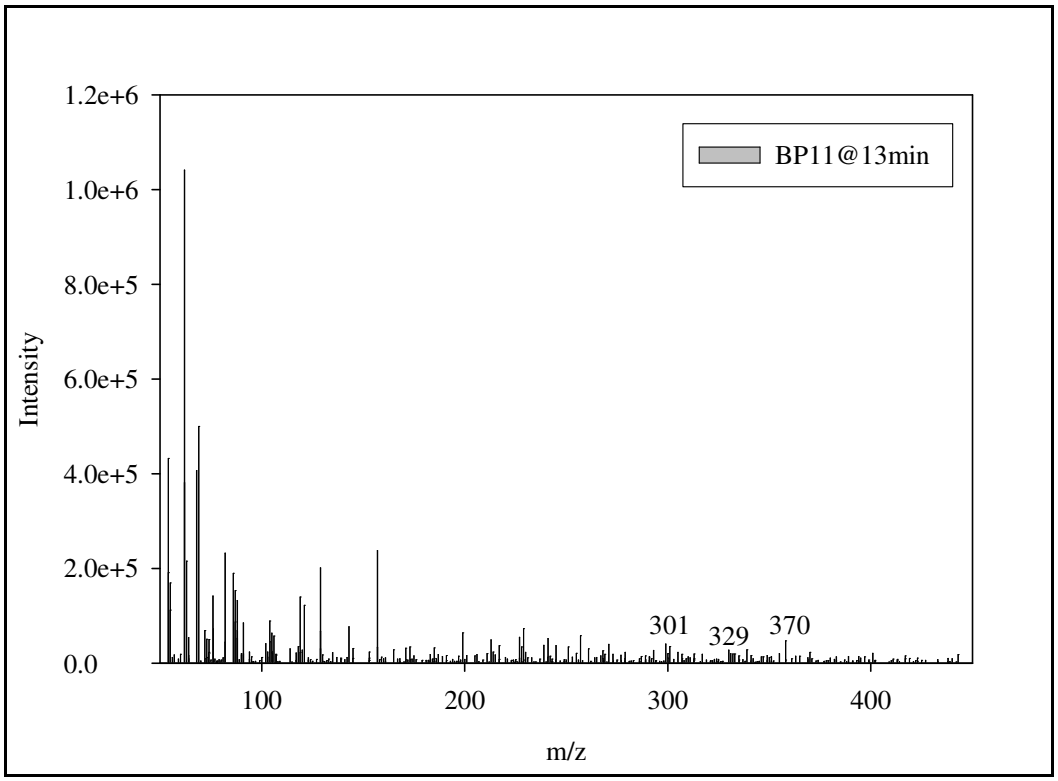


Figure B-55: Ozonation Byproducts of EE2 (ESI Positive)

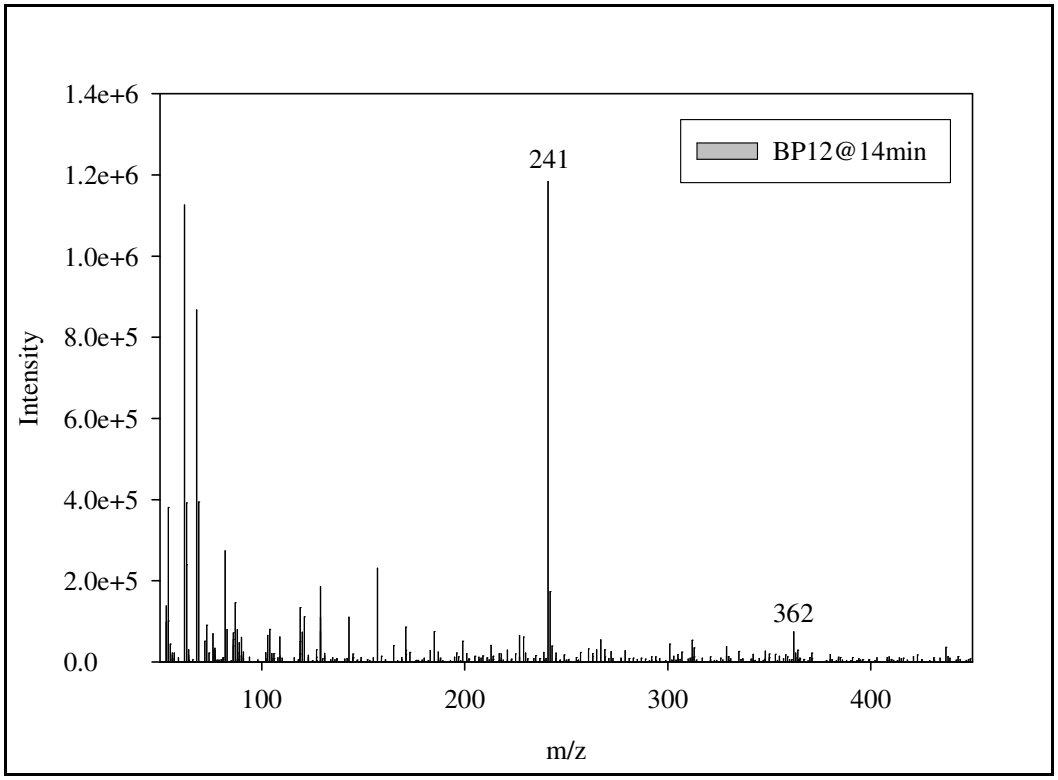


Figure B-56: Ozonation Byproducts of EE2 (ESI Positive)

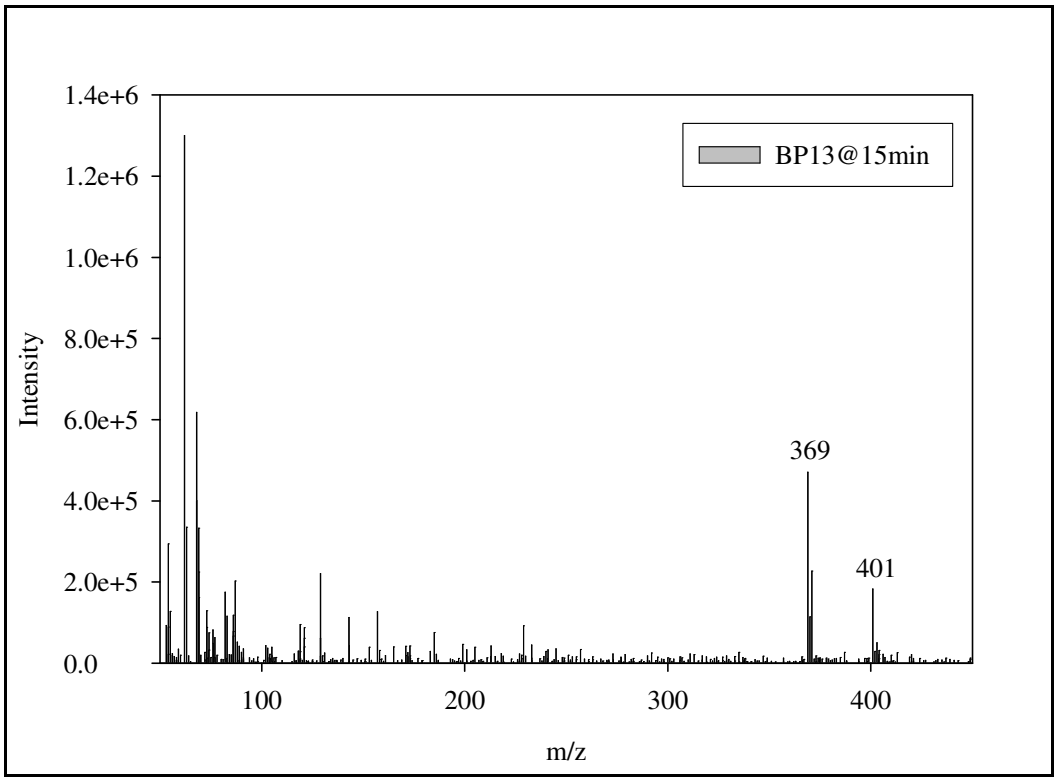


Figure B-57: Ozonation Byproducts of EE2 (ESI Positive)

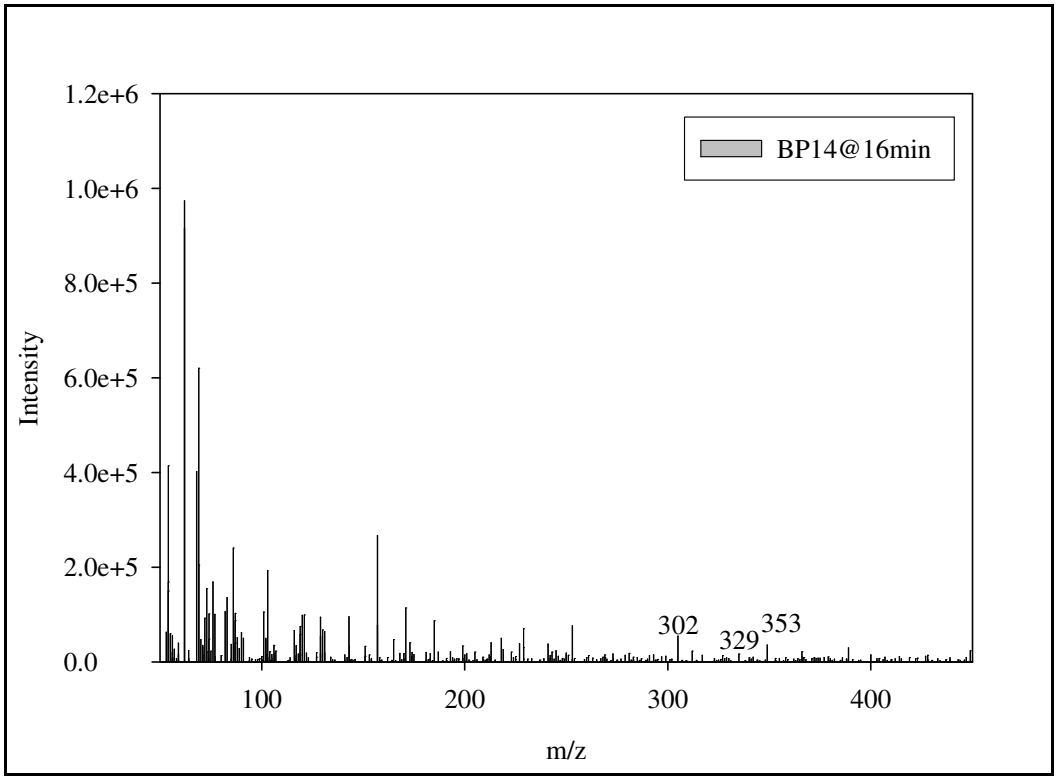


Figure B-58: Ozonation Byproducts of EE2 (ESI Positive)

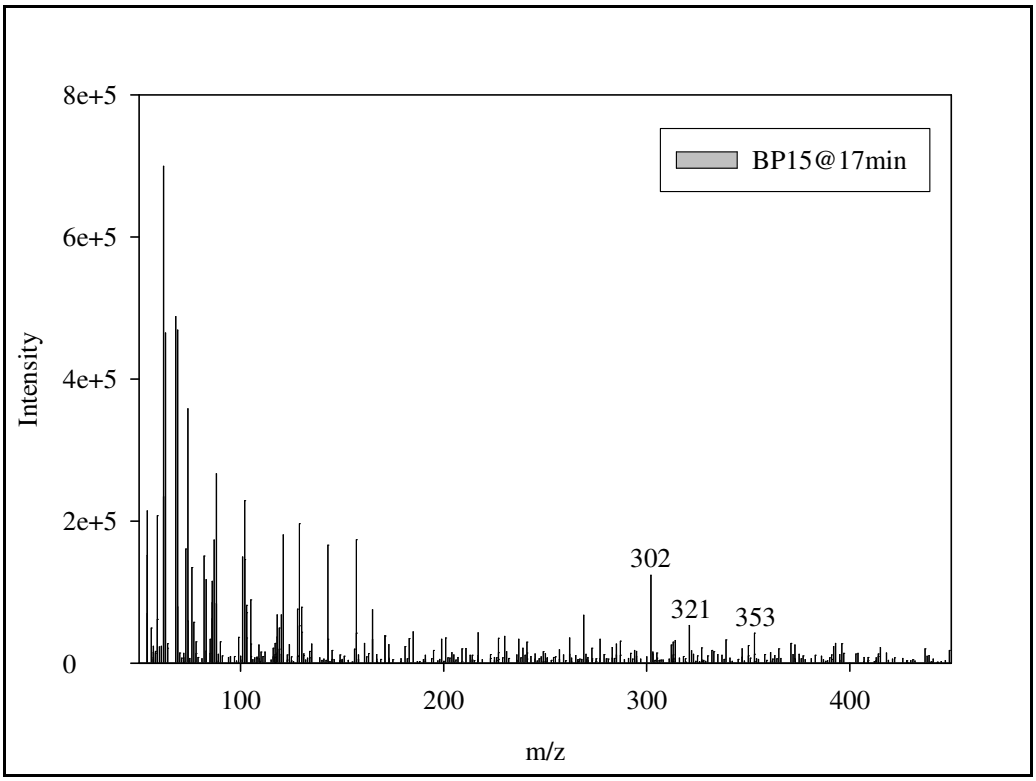


Figure B-59: Ozonation Byproducts of EE2 (ESI Positive)

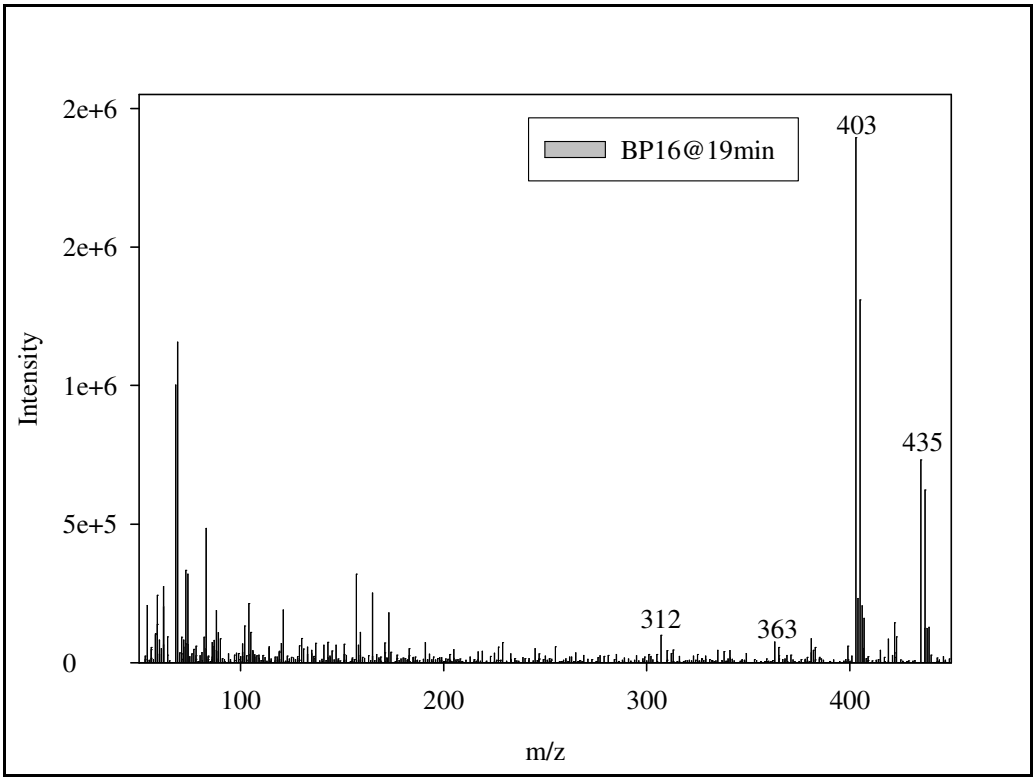


Figure B-60: Ozonation Byproducts of EE2 (ESI Positive)

Table B-19: Instantaneous Ozonation of DES (ESI Negative)

	% Total Peak Areas					
	1uM	**2uM**	**4uM**	**6uM**	**8uM**	**10uM**
DES	98.5	95.3	91.3	77.8	67.9	64.9
Byproduct 1 (8min)	0.1	0.1	0.1	0.3	0.4	0.4
Byproduct 2 (9min)	0.3	0.3	0.3	0.7	1.0	1.1
Byproduct 3 (11min)	0.2	0.2	0.2	0.4	0.3	0.3
Byproduct 4 (12min)	0.0	0.1	0.1	0.2	0.2	0.2
Byproduct 5 (13min)	0.1	0.1	0.1	0.2	0.2	0.3
Byproduct 6 (14min)	0.7	0.7	0.7	1.2	1.2	1.1
Byproduct 7 (21min)	0.2	0.1	0.1	0.0	0.1	0.1
Other Byproducts	0.0	3.2	7.1	19.3	28.7	31.6

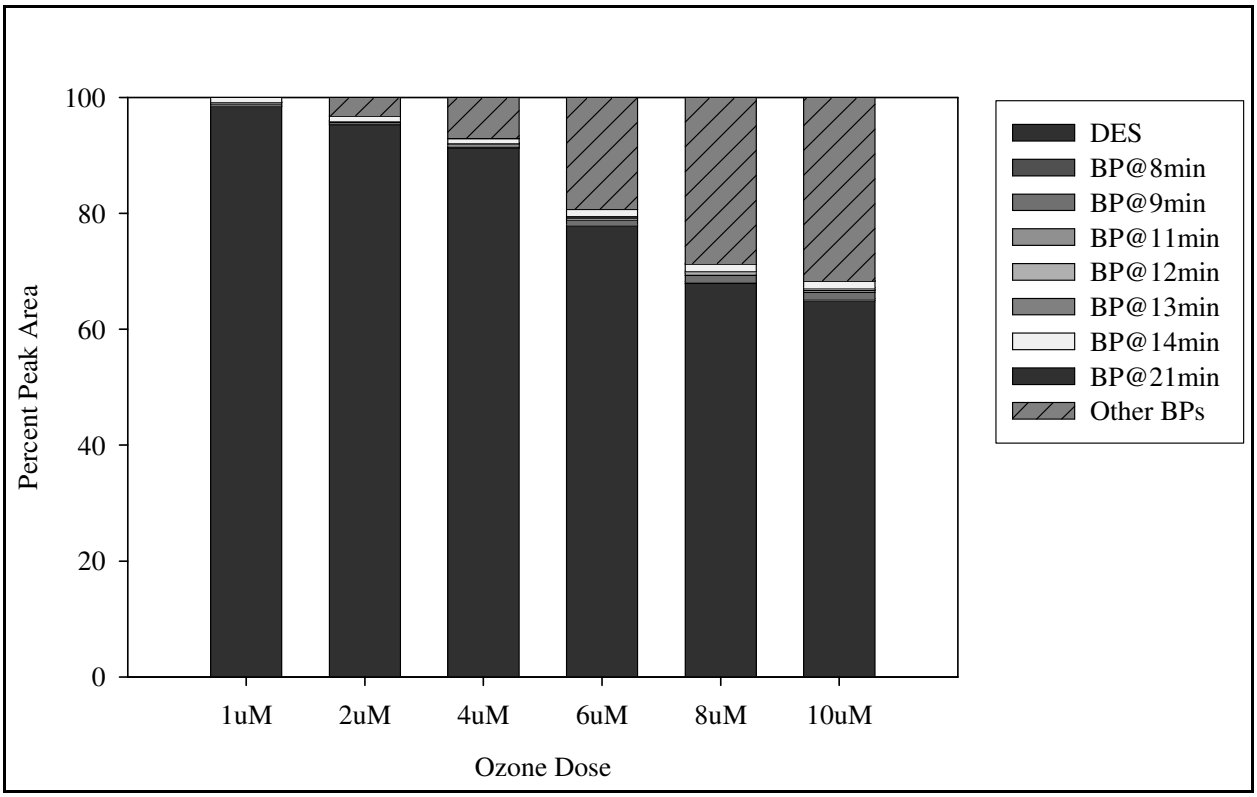


Figure B-61: Percent Peak Area for Ozonation Byproducts of DES (ESI Negative)

Table B-20: Instantaneous Ozonation of DES (ESI Positive)

	% Total Peak Areas					
	1uM	**2uM**	**4uM**	**6uM**	**8uM**	**10uM**
DES	93.0	93.5	92.6	83.3	82.8	62.8
Byproduct 1 (7min)	1.4	1.1	1.8	1.5	1.8	1.2
Byproduct 2 (8min)	0.6	0.7	0.4	0.6	0.7	0.9
Byproduct 3 (9min)	1.1	1.1	1.1	1.1	1.1	1.0
Byproduct 4 (10min)	0.1	0.1	0.2	0.2	0.2	0.5
Byproduct 5 (12min)	0.6	0.5	0.5	0.7	0.7	0.6
Byproduct 6 (13min)	0.2	0.2	0.2	0.4	0.4	0.4
Byproduct 7 (14min)	2.1	2.1	2.3	2.1	1.8	2.0
Byproduct 8 (16min)	0.3	0.1	0.3	2.3	3.8	0.2
Byproduct 9 (21min)	0.8	0.7	0.6	0.6	0.4	0.2
Other Byproducts	0.0	0.0	0.0	7.2	6.3	30.1

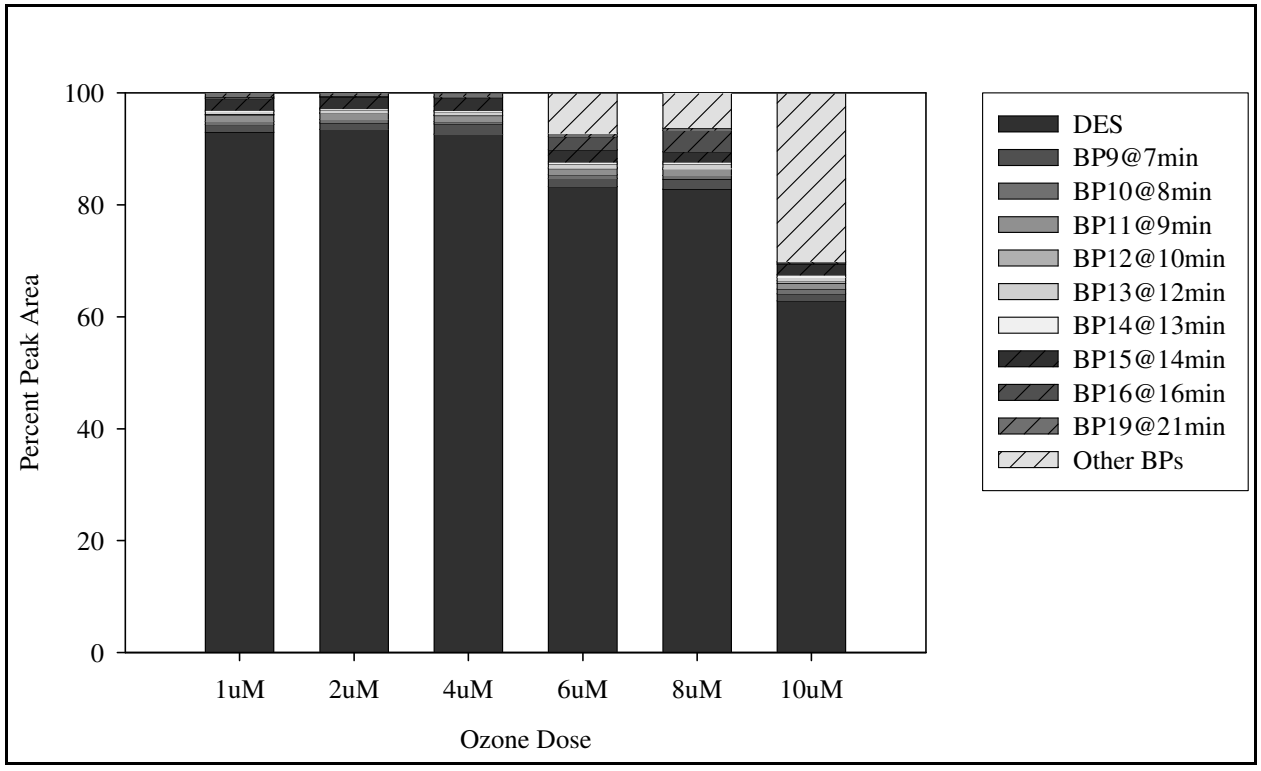


Figure B-62: Percent Peak Area for Ozonation Byproducts of DES (ESI Positive)

Table B-21: Short Term Ozonation of DES (ESI Negative)

	% Total Peak Areas			
	1.5mg/L	**2.0mg/L**	**3.0mg/L**	**6mg/L**
DES	16.8	5.9	0.9	0.0
Byproduct 1 (8min)	0.3	0.5	0.5	0.2
Byproduct 2 (9min)	4.5	8.7	9.3	1.6
Byproduct 3 (11min)	0.3	0.3	0.2	0.0
Byproduct 4 (12min)	0.3	0.4	0.6	0.4
Byproduct 5 (13min)	0.2	0.3	0.2	0.0
Byproduct 6 (14min)	0.3	0.5	0.4	0.0
Byproduct 7 (16min)	5.6	5.6	0.0	0.0
Byproduct 8 (19min)	0.9	0.7	0.0	0.0
Other Byproducts	70.7	77.2	87.8	97.7

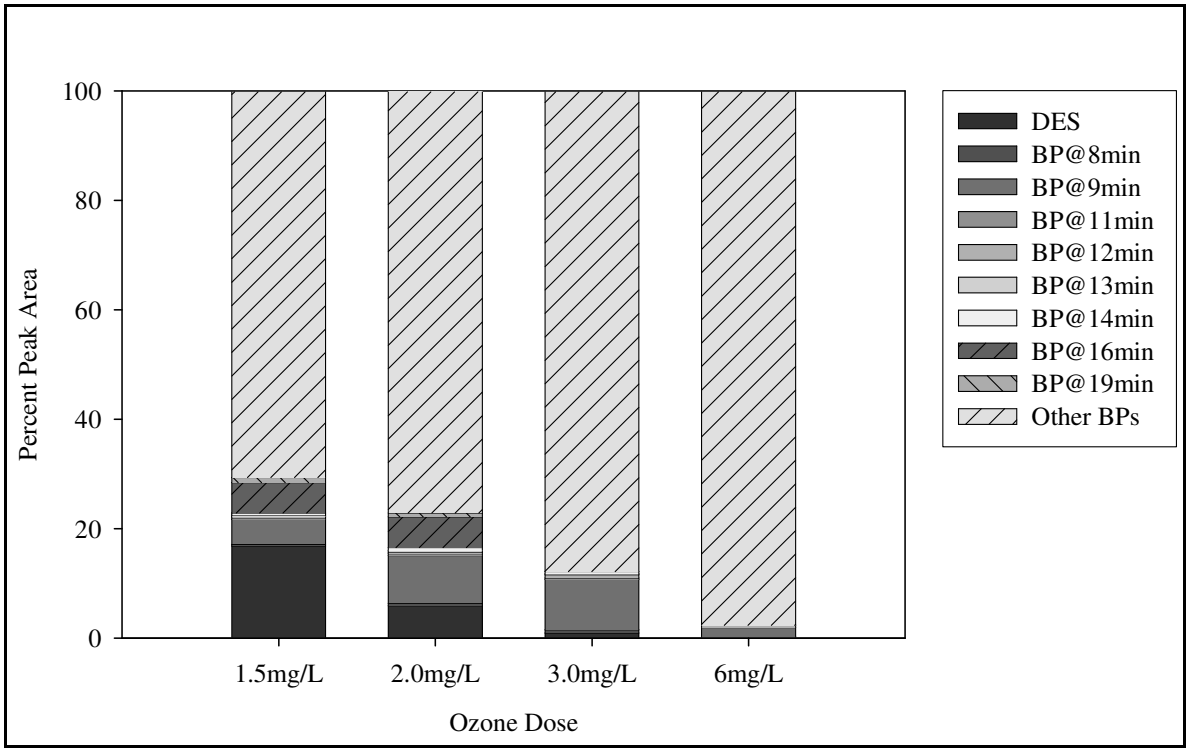


Figure B-63: Percent Peak Area for Short Term Ozonation Byproducts of DES (ESI Negative)

Table B-22: Short Term Ozonation of DES (ESI Positive)

	% Total Peak Areas			
	1.5mg/L	**2.0mg/L**	**3.0mg/L**	**6mg/L**
DES	13.1	3.9	0.6	0.0
Byproduct 9 (7min)	1.1	1.5	1.0	1.2
Byproduct 10 (8min)	0.9	0.4	0.5	0.3
Byproduct 11 (9min)	3.2	5.5	7.9	2.0
Byproduct 12 (10min)	0.3	0.2	0.3	0.2
Byproduct 13 (12min)	0.3	0.5	0.6	0.6
Byproduct 14 (13min)	0.3	0.3	0.3	0.0
Byproduct 15 (14min)	0.9	0.9	0.6	0.0
Byproduct 16 (15min)	5.0	6.6	5.4	1.7
Byproduct 17 (17min)	5.4	0.6	0.2	0.0
Byproduct 18 (19min)	0.5	0.3	0.1	0.0
Byproduct 19 (21min)	0.3	0.3	0.2	0.0
Other Byproducts	68.7	78.9	82.3	94.1

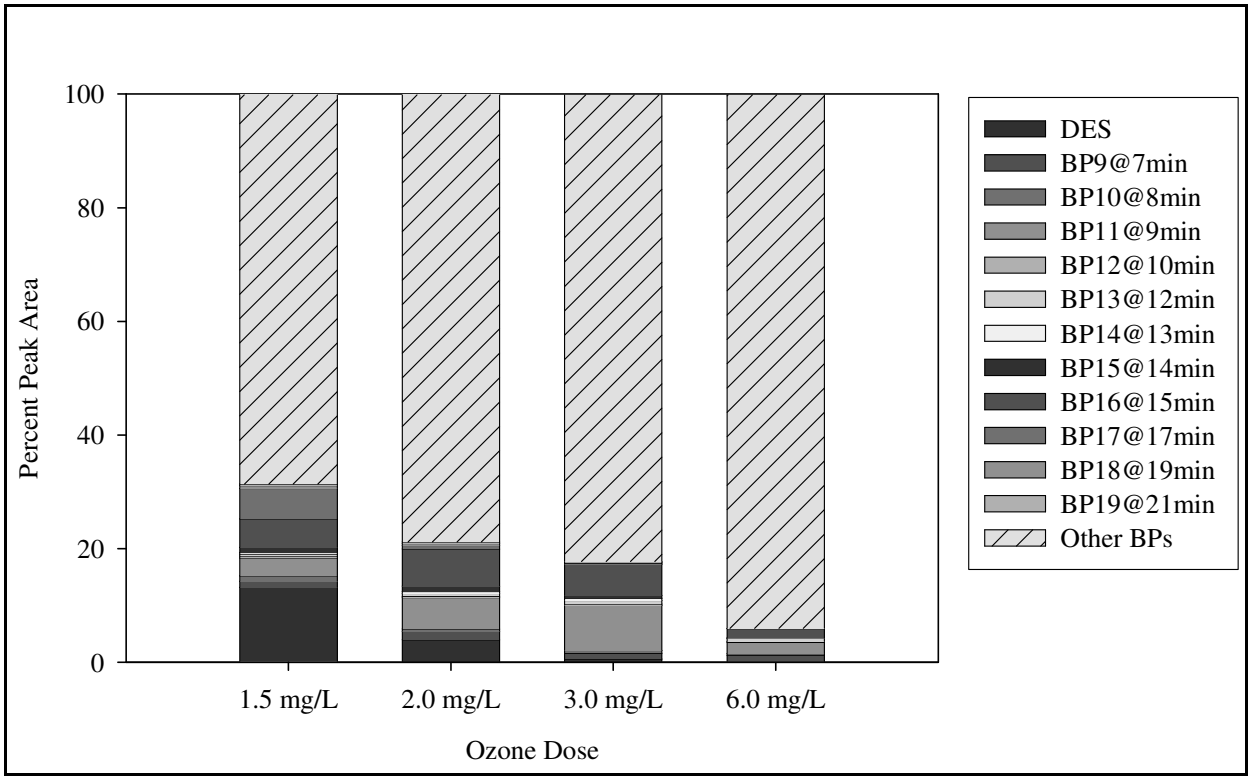


Figure B-64: Percent Peak Area for Short Term Ozonation Byproducts of DES (ESI Positive)

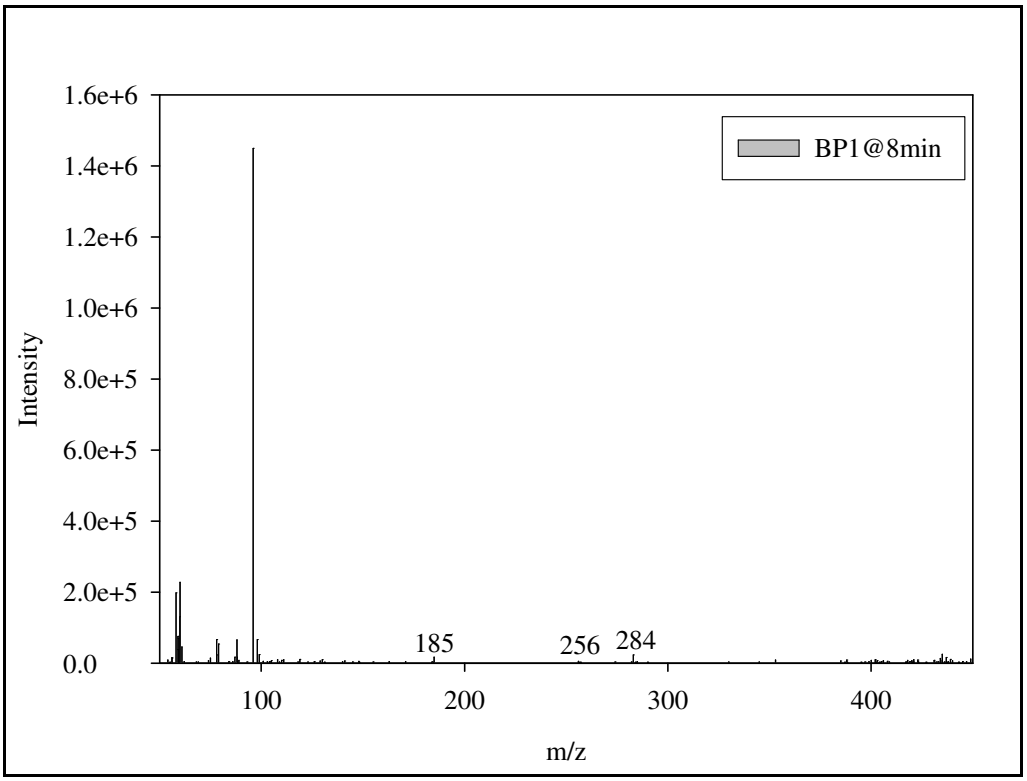


Figure B-65: Ozonation Byproduct of DES (ESI Negative)

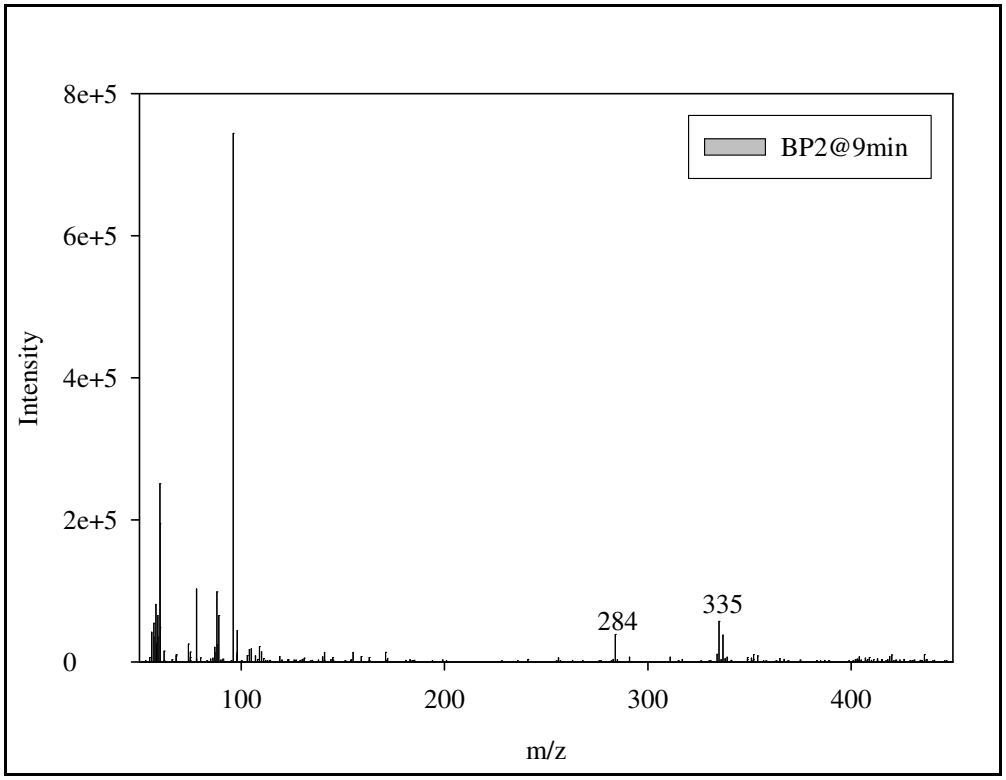


Figure B-66: Ozonation Byproduct of DES (ESI Negative)

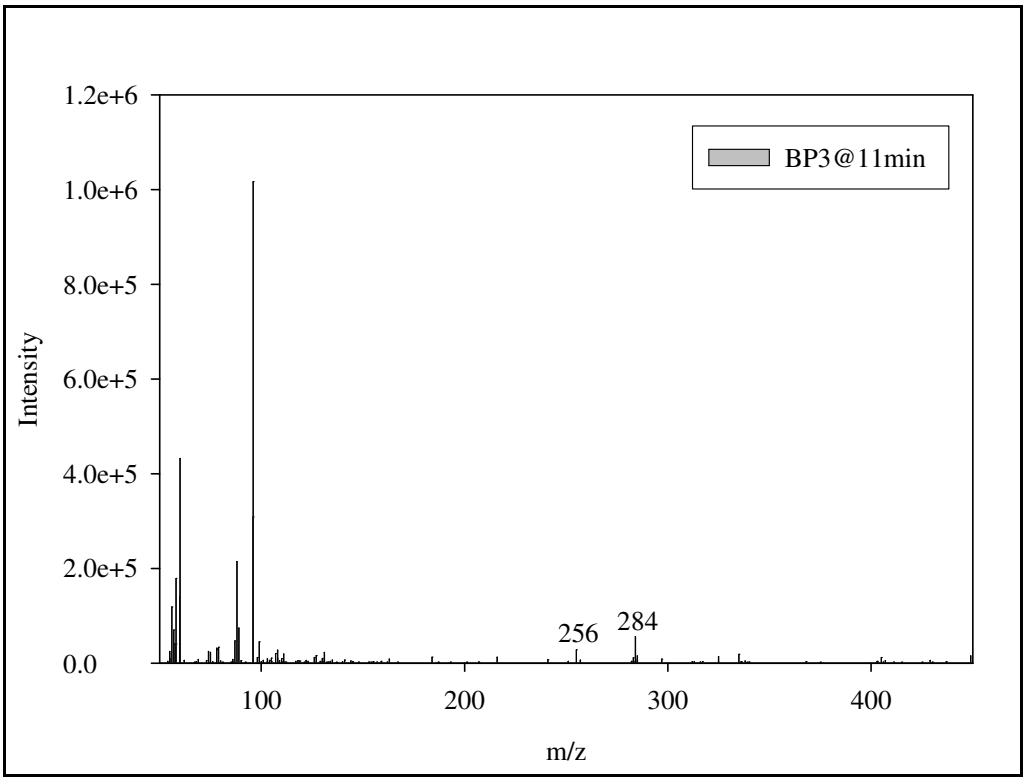


Figure B-67: Ozonation Byproduct of DES (ESI Negative)

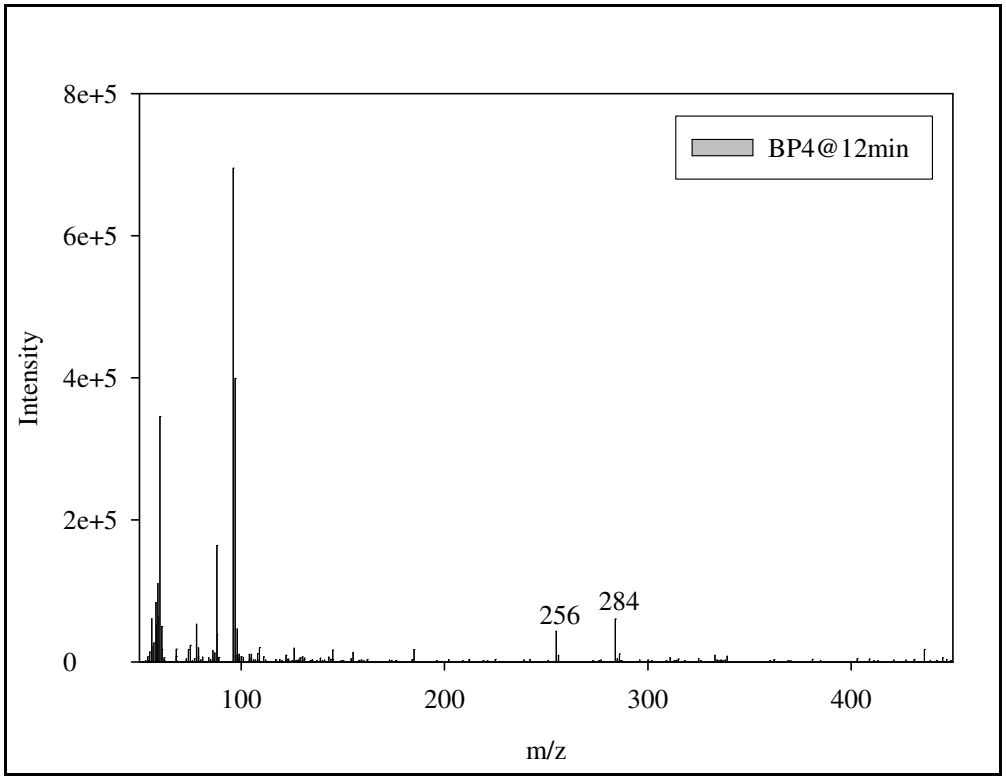


Figure B-68: Ozonation Byproduct of DES (ESI Negative)

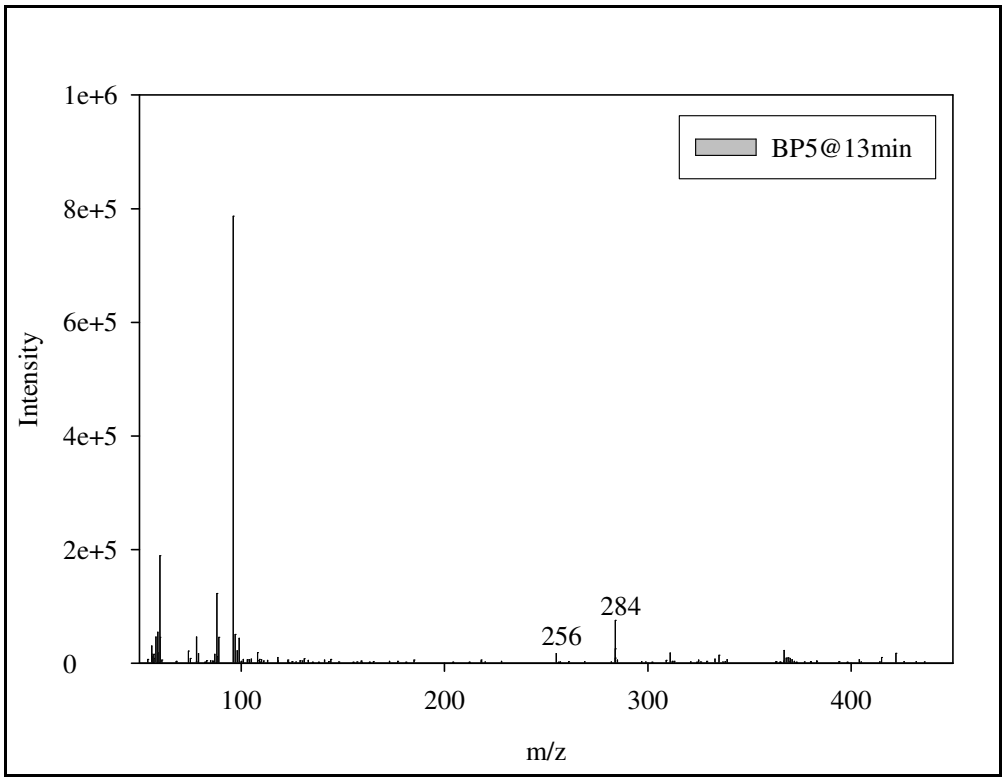


Figure B-69: Ozonation Byproduct of DES (ESI Negative)

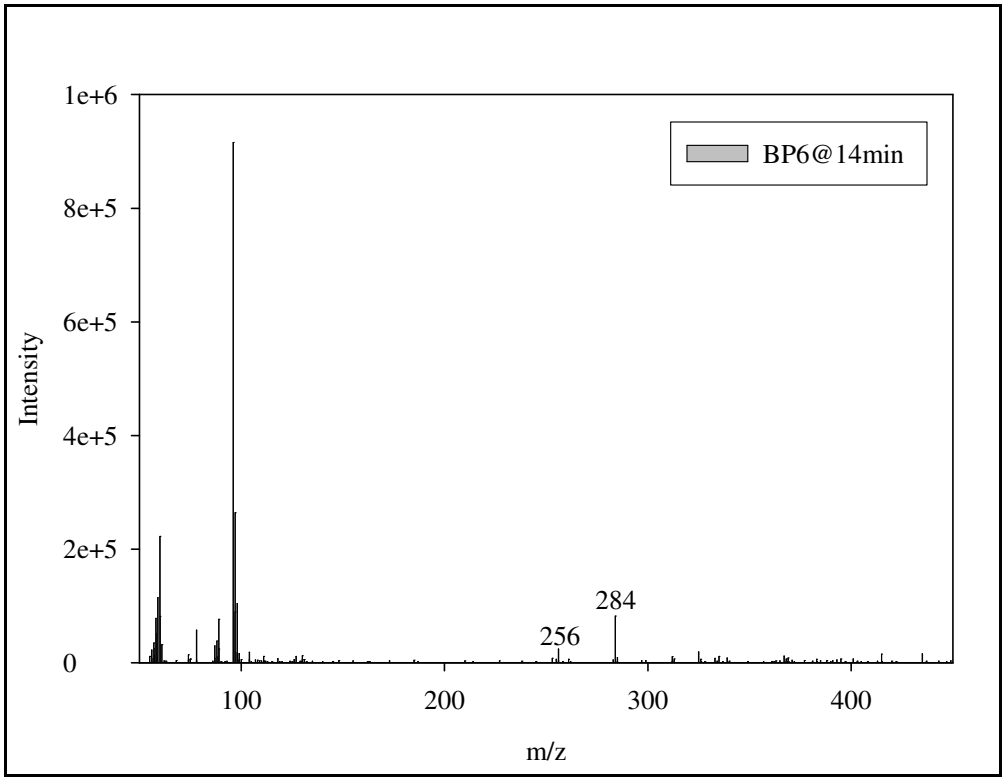


Figure B-70: Ozonation Byproduct of DES (ESI Negative)

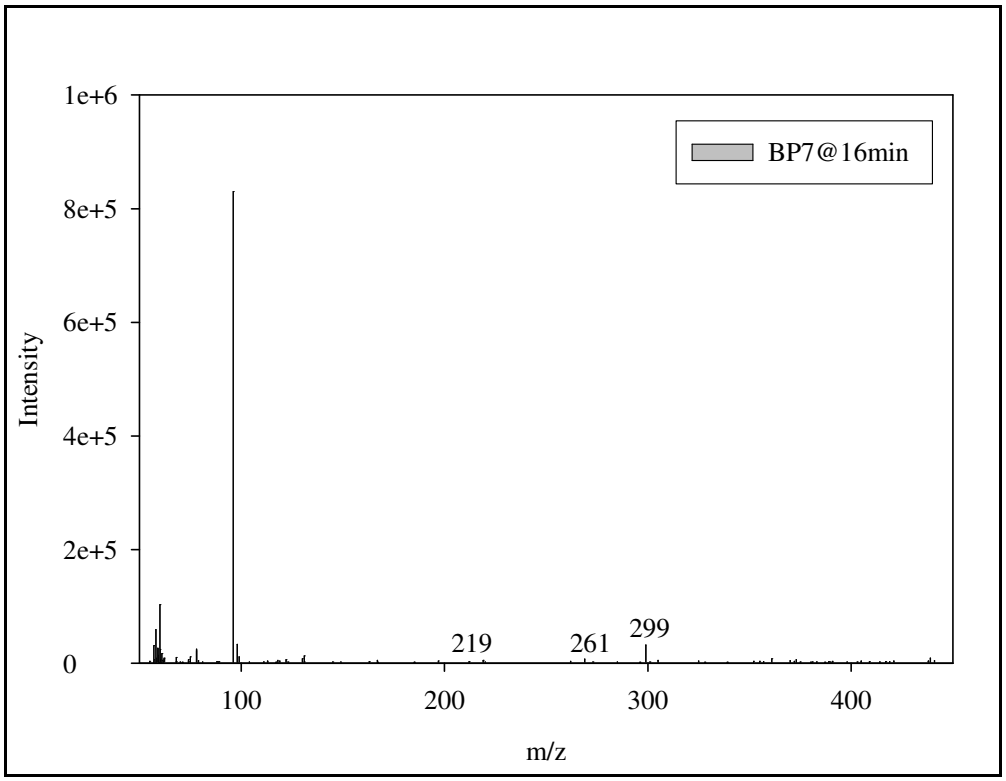


Figure B-71: Ozonation Byproduct of DES (ESI Negative)

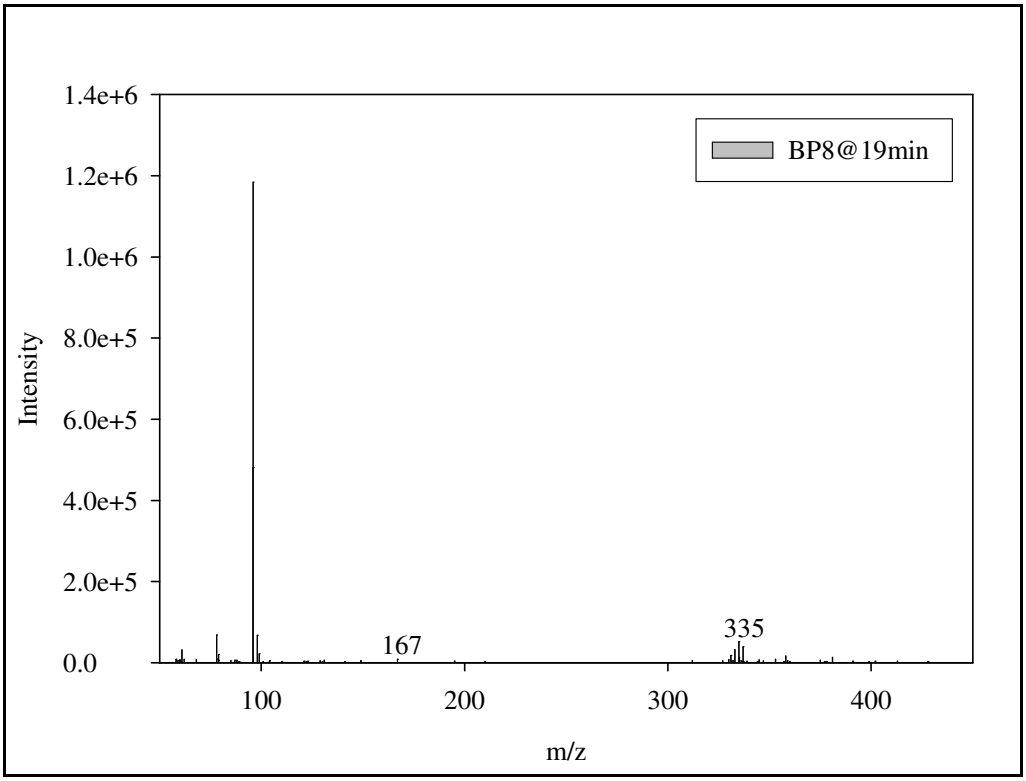


Figure B-72: Ozonation Byproduct of DES (ESI Negative)

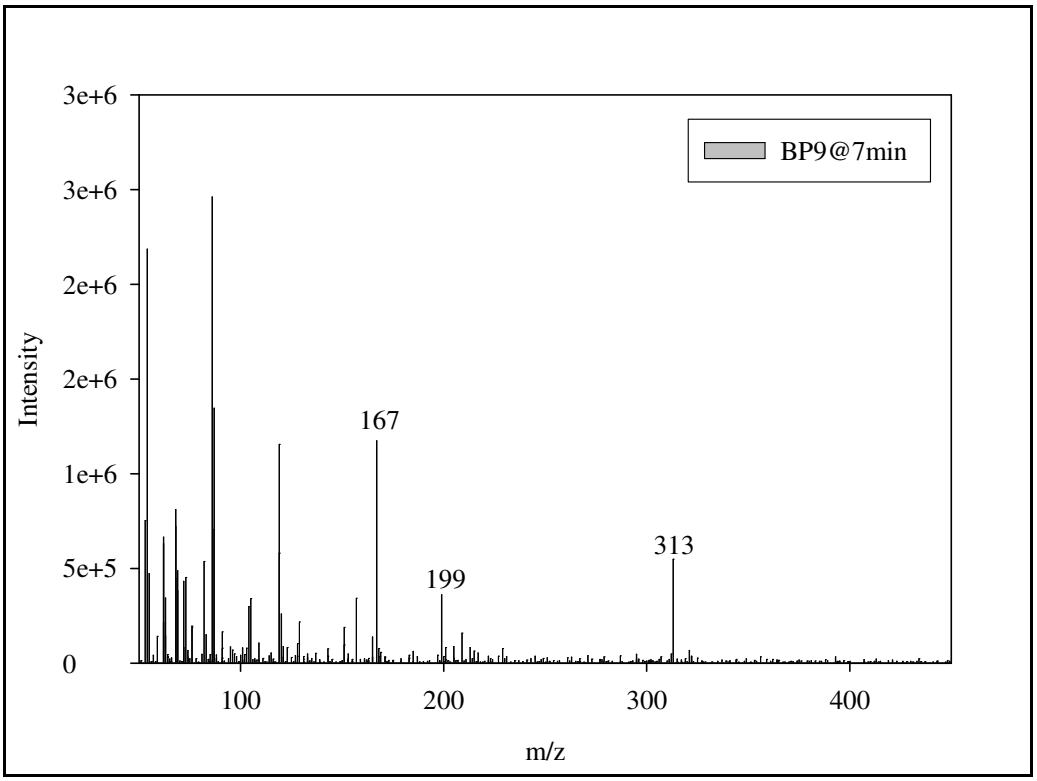


Figure B-73: Ozonation Byproduct of DES (ESI Positive)

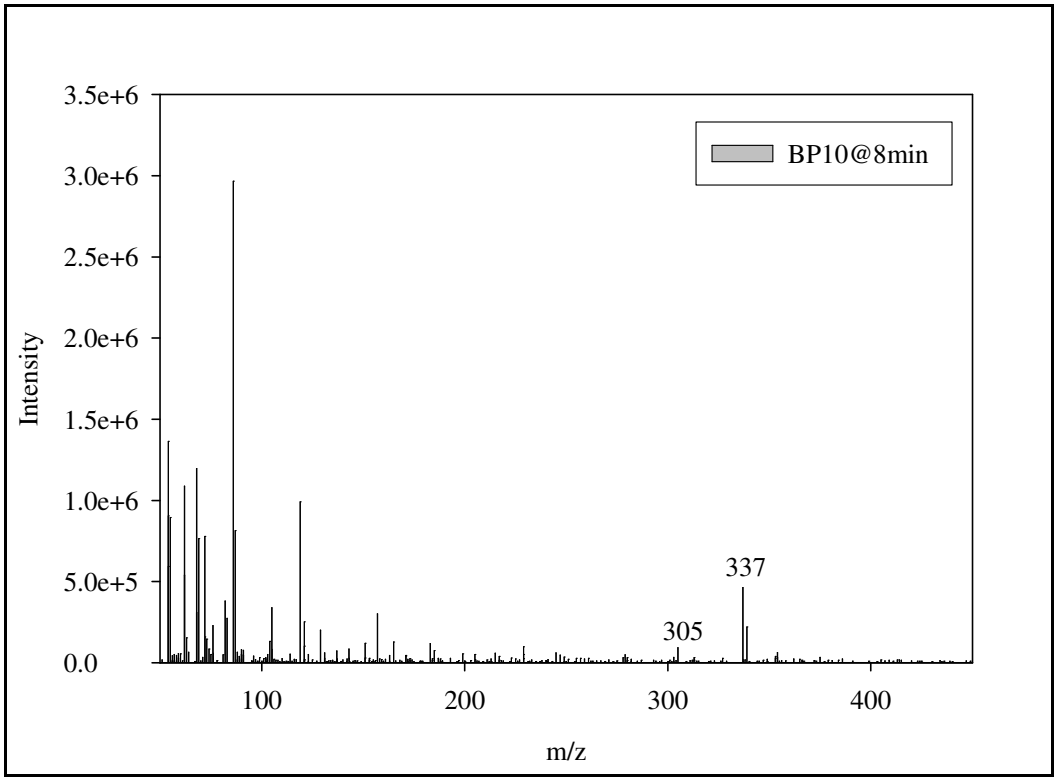


Figure B-74: Ozonation Byproduct of DES (ESI Positive)

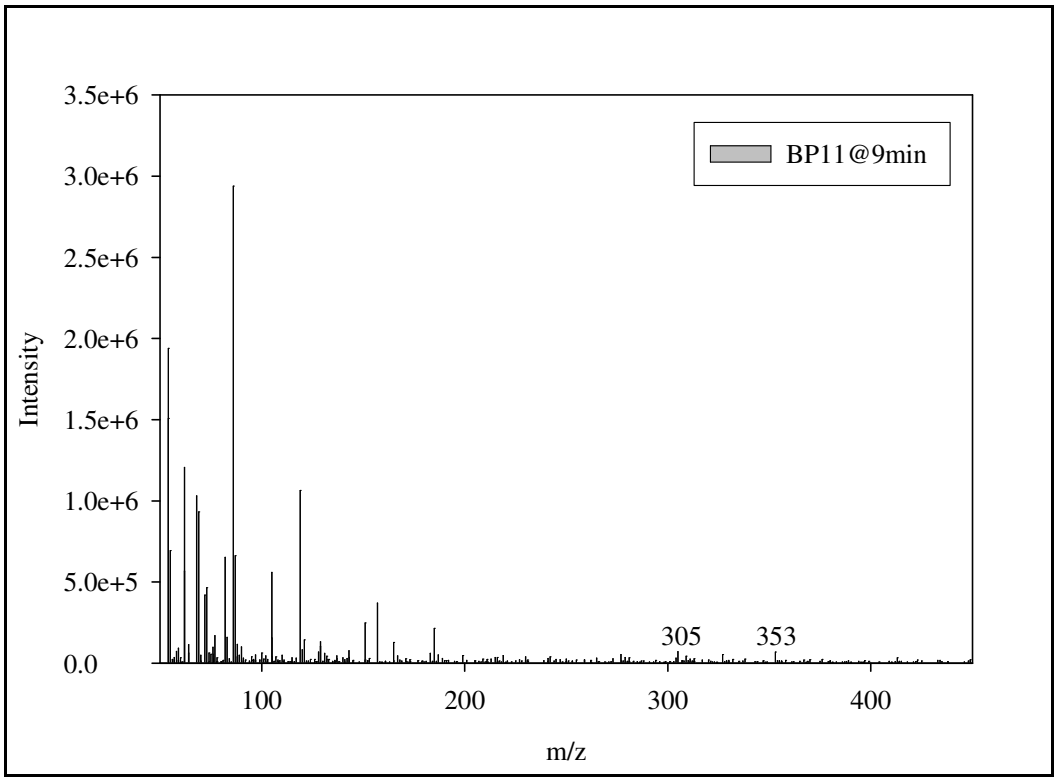


Figure B-75: Ozonation Byproduct of DES (ESI Positive)

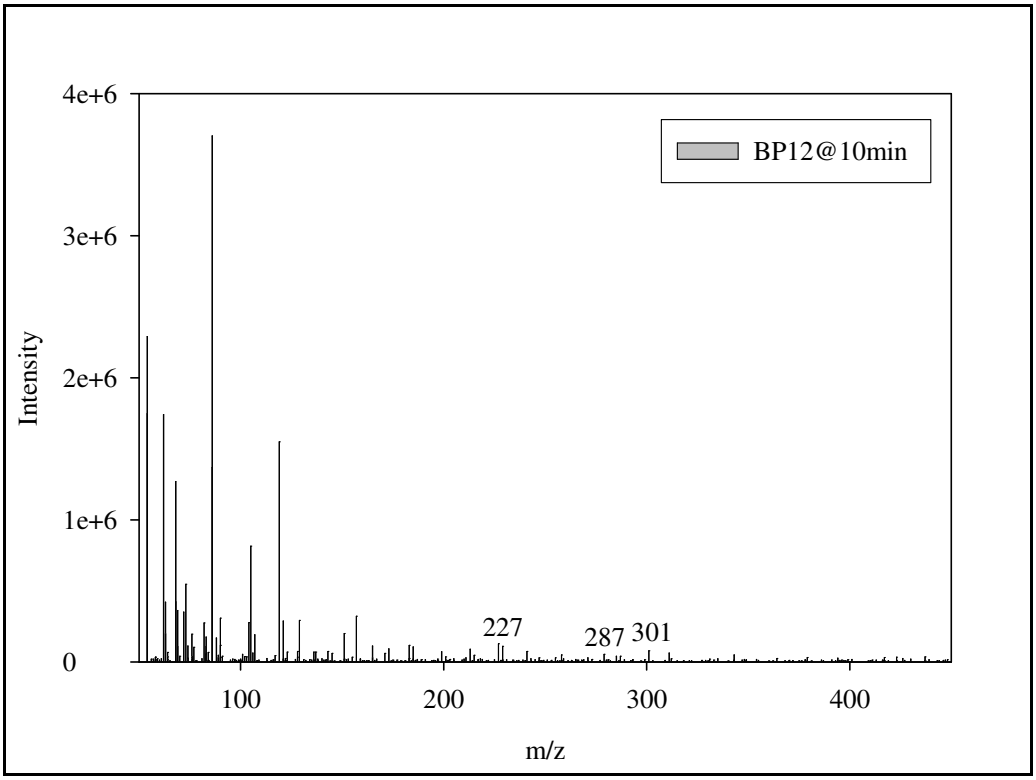


Figure B-76: Ozonation Byproduct of DES (ESI Positive)

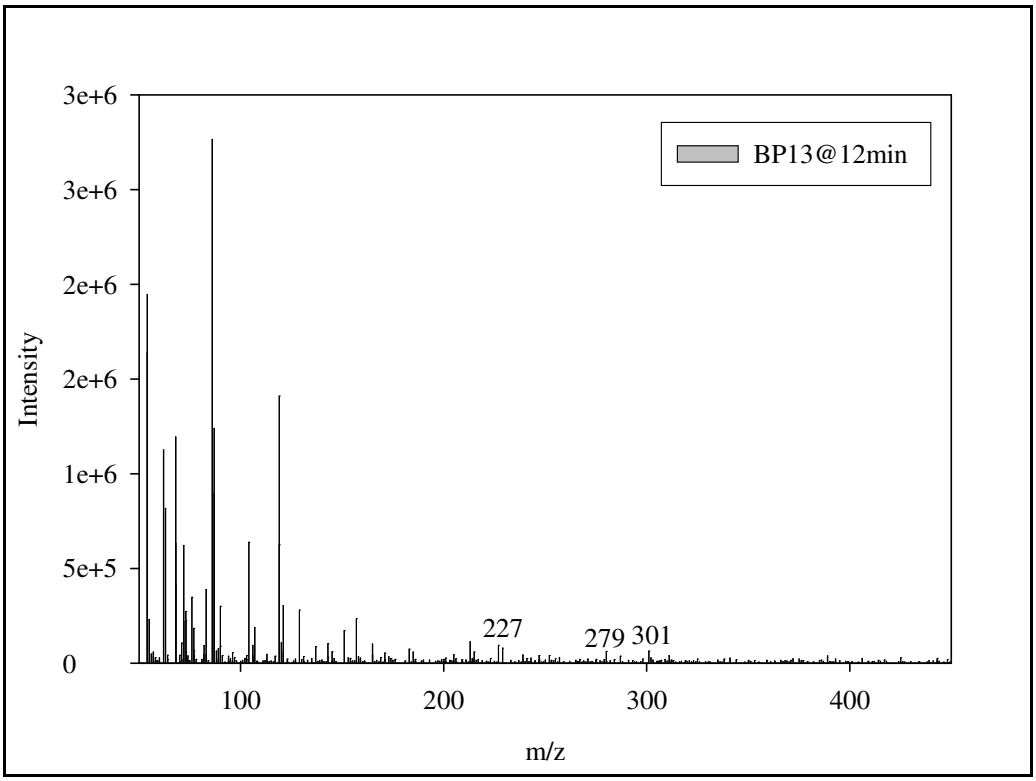


Figure B-77: Ozonation Byproduct of DES (ESI Positive)

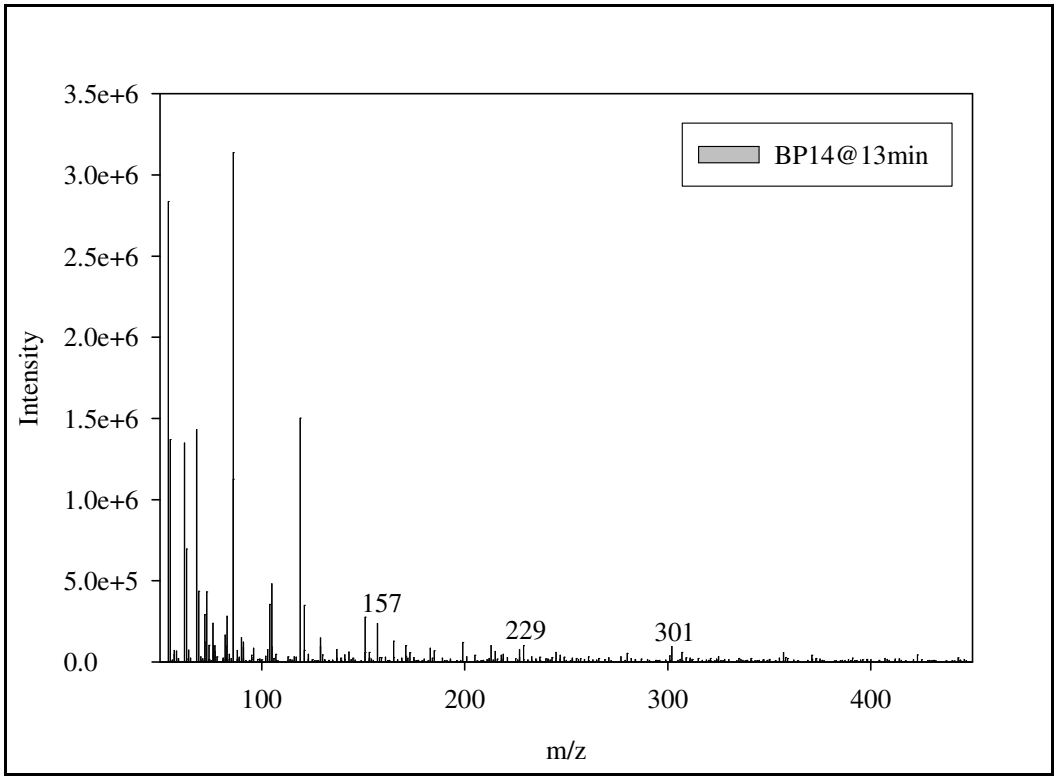


Figure B-78: Ozonation Byproduct of DES (ESI Positive)

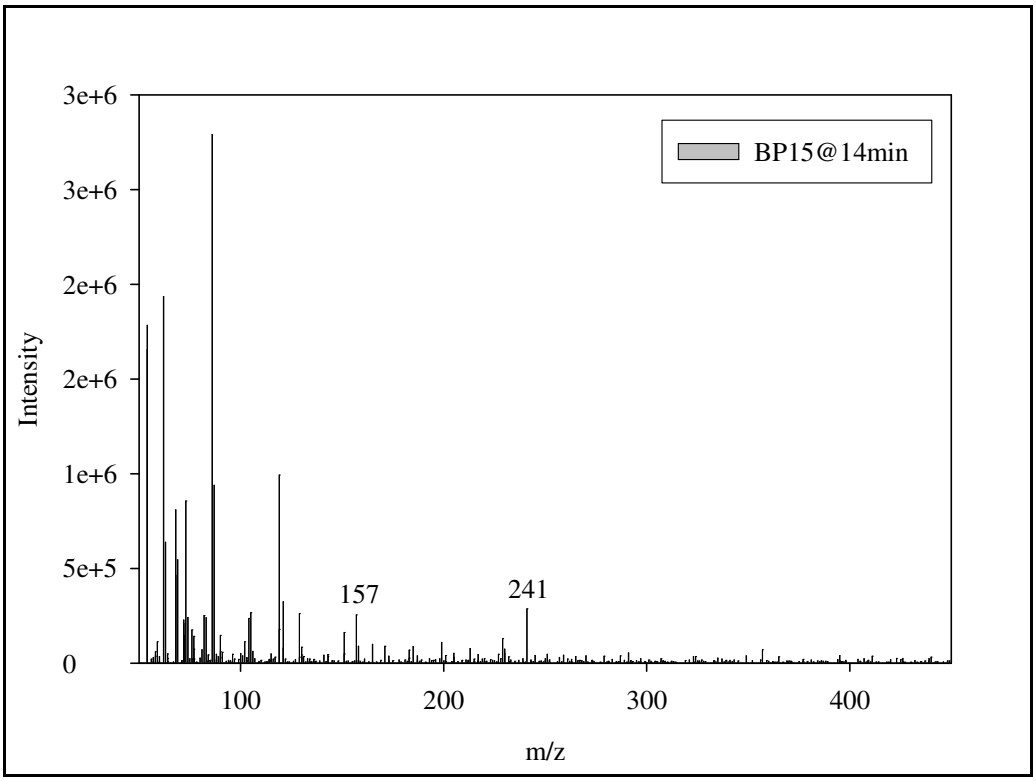


Figure B-79: Ozonation Byproduct of DES (ESI Positive)

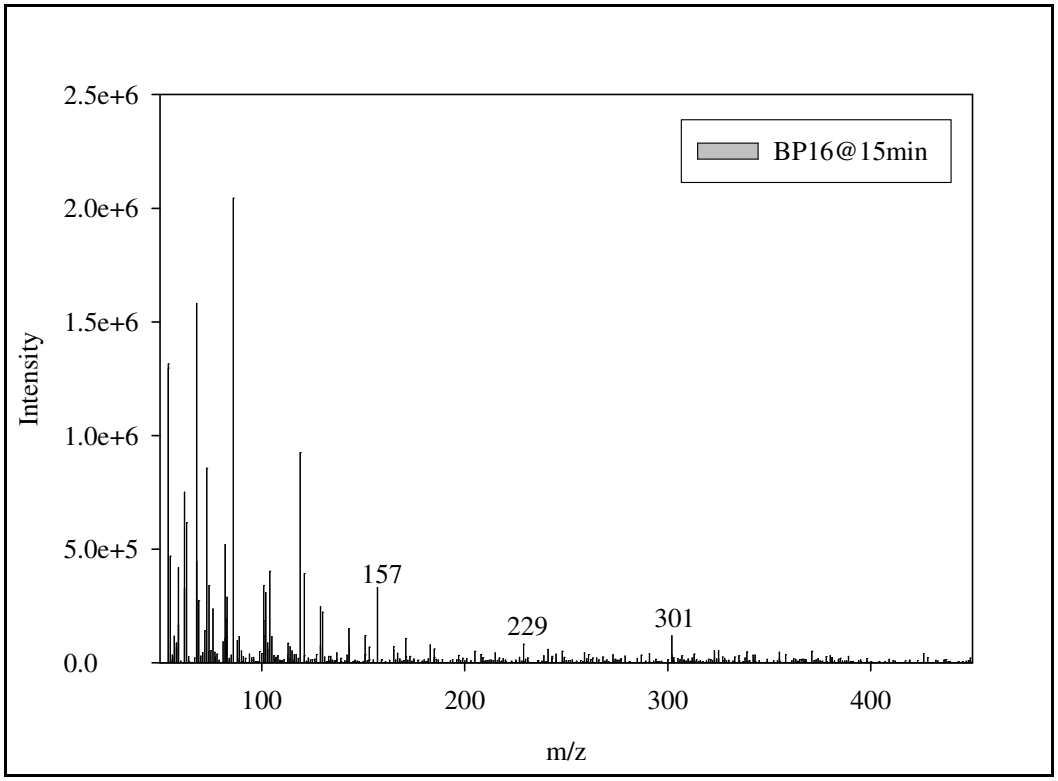


Figure B-80: Ozonation Byproduct of DES (ESI Positive)

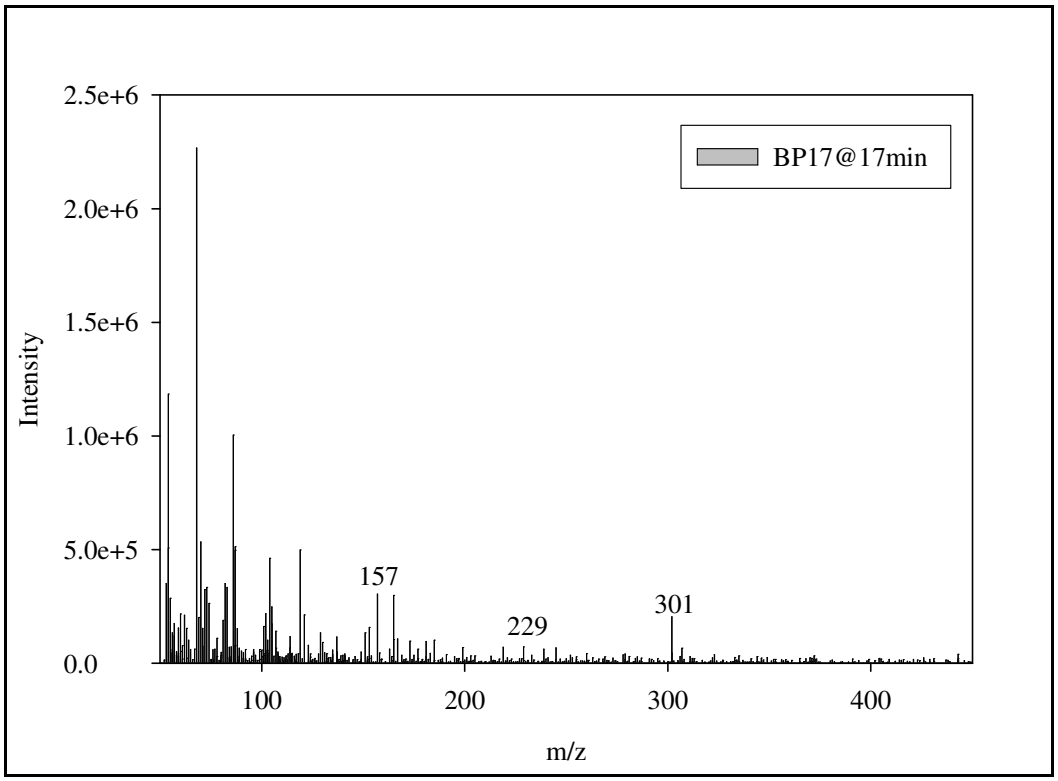


Figure B-81: Ozonation Byproduct of DES (ESI Positive)

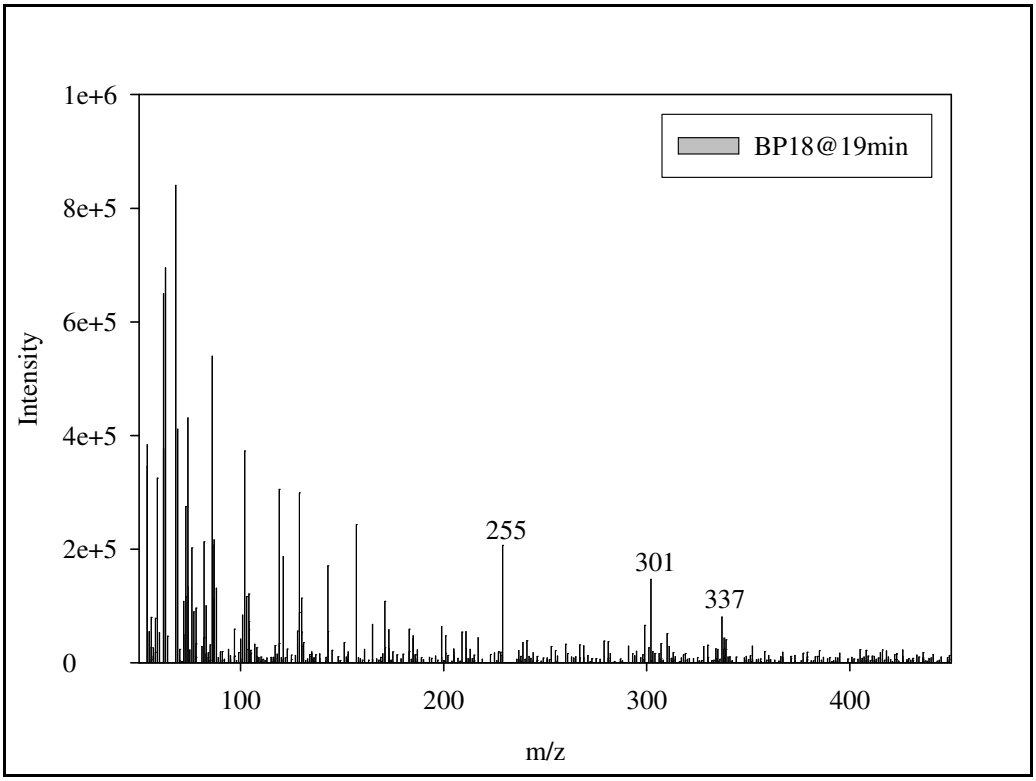


Figure B-82: Ozonation Byproduct of DES (ESI Positive)

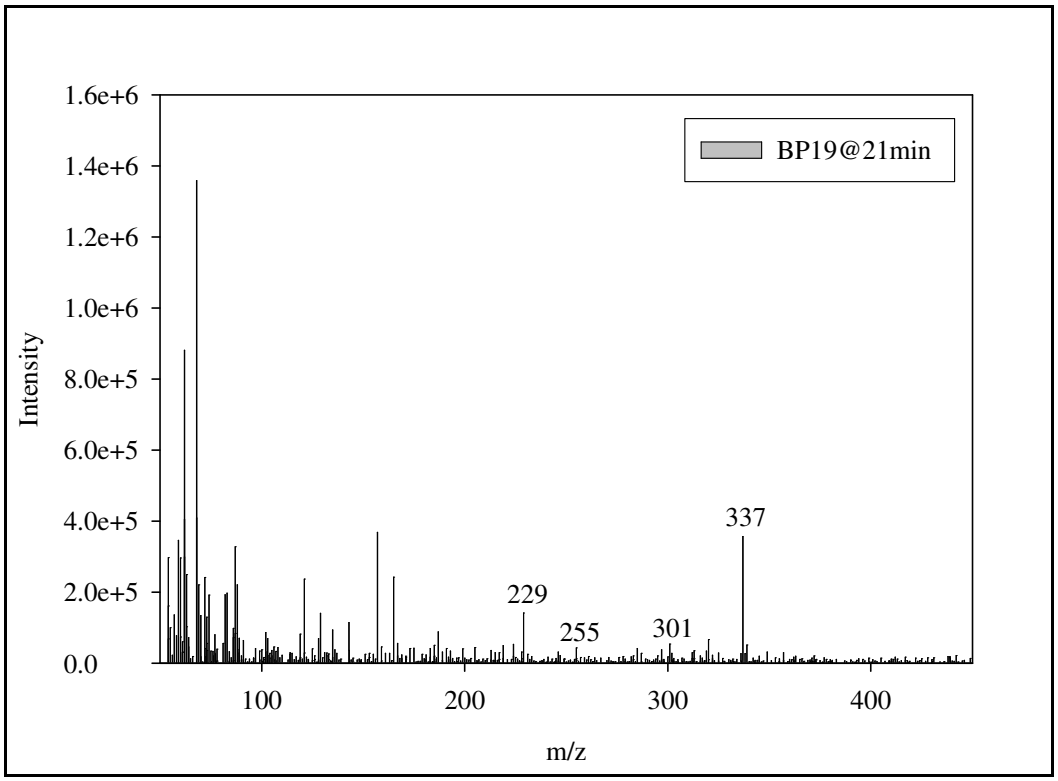


Figure B-83: Ozonation Byproduct of DES (ESI Positive)

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